TRANSCRIPTOMIC PROFILING OF VASCULAR ENDOTHELIAL GROWTH FACTOR-INDUCED SIGNATURE GENES IN HUMAN CERVICAL EPITHELIAL CELLS

A Thesis
by
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Abstract

TRANSCRIPTOMIC PROFILING OF VASCULAR ENDOTHELIAL GROWTH FACTOR-INDUCED SIGNATURE GENES IN HUMAN CERVICAL EPITHELIAL CELLS

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Cervical epithelia cells play central roles in cervical remodeling (CR) during pregnancy and cervical events during the menstrual cycle, including mounting physical and immunological barriers, proliferation and differentiation, maintenance of fluid balance and likely in withstanding the mechanical force exerted by the growing fetus prior to term. We have previously characterized the cellular localization of vascular endothelial growth factor (VEGF), its receptor and signaling molecules in the cervix of rodents, its profile over the course of pregnancy and immediately after birth, as well as characterized its genome- and proteome-wide signature genes/proteins. These earlier studies reveal that VEGF and its associated molecules largely target and are localized in the cervical epithelial cells. For this reason, in the present study, we attempt to decipher the specific roles of VEGF in Human cervical epithelial cells by delineating VEGF signature genes using RNA sequencing in order to characterize the specific biological effects of VEGF in these cells.

Specifically, following optimization of dosage and incubation time, Human cervical epithelial cells were treated with either vehicle only (culture media, i.e., negative control) or with 50 ng of exogenous VEGF protein (i.e., treatment group) using an *in vitro* model. Following treatment, cells were harvested, total RNA extracted, followed by RNA sequencing, which was verified using real-time PCR analyses of selected genes. Out of a total 25,000 genes that were screened, 162 genes were found to be differentially expressed in Human cervical epithelial cells, of which 12 genes were found to be statistically *significantly* differentially expressed. The differentially expressed genes (162) were categorized by biological function, which included **1**) proliferation, **2**) immune response, **3**) structure/matrix, **4**) mitochondrial function, **5**) cell adhesion/communication, **6**) pseudogenes, **7**) non-coding RNA, **8**) miscellaneous genes and **9**) uncharacterized genes. We conclude that VEGF plays a key role in CR by altering the expression of genes that regulate proliferation, immune response, energy metabolism and cell structure, biological processes that are essential to CR.

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First and foremost, I would like to express my sincere appreciation for both of my parents, Robin and Steve Johnson. They have been my constant support throughout life, and I would not be the individual I am today without their guidance and love.

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Finally, I am grateful to the Office of Student Research at Appalachian State University for their monetary support of this project.

Dedication

I would like to dedicate this thesis to both of my grandmothers, Cloyie Dolinger and Meryl Queen. Two ladies, who have each inspired my passion for women's health in different ways. I could not have asked for any more sincerely genuine and passionate individuals to guide me through life.

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Chapter One: Introduction

Preterm birth (PTB) has a significant impact on babies and their families, as well as the national healthcare system. PTB is considered birth before 37 weeks of pregnancy and the leading cause of death in children 5 years old or younger world-wide (Blencowe *et al*. 2013). Approximately 15 million babies are born premature every year, and of these about 1 million die from PTB complications annually (Blencowe *et al*. 2013, WHO 2018). Furthermore, on average the cost associated with preterm birth is $55,000 for the first year of the baby's life compared to approximately $5,000 for a healthy, full-term baby in the first year (March of Dimes, 2014). These costs may, in part, be due to the fact that preterm infants stay an average of 13 days in the hospital compared to a fetus carried to term whose average hospital stay is only 1.5 days (Purisch & Gyamfi-Bannerman, 2017). Also, fetuses born preterm are at a higher risk for death or health complications, including, but not limited to, breathing problems, feeding difficulties, cerebral palsy, developmental delays, vision and hearing problems (CDC 2018). Currently, PTB has an estimated annual health care cost of $31 billion USD per year in the USA (Phibbs & Schmitt 2006, Caughey *et al*. 2016).

Preterm birth is primarily associated with aberrations in the uterus and cervix and can be categorized in 3 ways: spontaneous labor, preterm premature rupture of membranes (PPROM), or labor induction for maternal or fetal indications (Goldenberg *et al*. 2008). The specific causes of preterm labor are complex and multifactorial and include: infection, inflammation, vascular disease, pre-eclampsia or eclampsia, intrauterine growth restriction and aberrations in cervical remodeling (CR) (Goldenberg *et al*. 2008).

Of particular interest to the present study is the role that CR plays in the induction of PTB. CR is a progressive process characterized by structural and biochemical alterations that take place in the cervix over the course of pregnancy and immediately after (Vink & Feltovich, 2016). It is divided into four overlapping phases: softening, ripening, dilation, and repair (Vink & Feltovich, 2016). Cervical softening is characterized by collagen reorganization, growth, increased vascularization and edema (Word *et al.* 2007). Cervical ripening generally entails the period prior to uterine contractions and is characterized by an increase in abundance of proteoglycan, glycosaminoglycan, collagen synthesis, and an increase in cell proliferation (Lee *et al*. 2005, Word *et al.* 2007). Cervical dilation occurs during active labor and is characterized by the presence of leukocytes and release of proteases and collagenases into the extracellular matrix (Word *et al.* 2007). The final phase of cervical remodeling is termed repair and involves a decrease in inflammation, tissue dehydration, and re-organization of the structural integrity of the cervical tissue (Word *et al.* 2007). Cervical repair occurs immediately after parturition (Word *et al.* 2007). Of particular interest to the present study is the ripening phase of CR when cervical growth and proliferation largely occur (Mahendroo *et al*. 1999, Read *et al*. 2007, Ruscheinsky *et al*. 2008, McGee *et al*. 2017). The growth of cervical tissue during this phase (cervical ripening) is crucial for the cervix to withstand intrauterine pressure and gravity exerted by the growing fetus (Myers *et al.* 2015). Furthermore, cervical epithelial cells also undergo cellular differentiation, which is essential for performing its varied roles at different points of the menstrual cycle and pregnancy, such as maintaining the immunological and permeability barrier, as discussed later (Timmons *et al*. 2007).

Understanding the physical and physiological complexity of the cervix is essential in the study of CR. Structurally, the cervix is a barrel-shaped structure located between the vagina and the uterus in the female reproductive tract, and is divided into two functionally distinct portions, namely the endocervix and ectocervix. These two cervical divisions (endocervix and ectocervix) are connected by a squamo-columnar junction (SCJ) or transformation zone (TZ), which is a narrow and contiguous junction comprised of metaplastic squamous cells derived from stem cells of the endocervix (Vassilakos *et al*. 2017, Deng *et al*. 2018). Cervical tissue is comprised of a variety of cell types (Feltovich & Carlson, 2017), which include smooth muscle, fibroblasts, vascular, immune and epithelial cells, which are embedded in the extracellular matrix (ECM). The ECM itself is comprised of proteins and proteoglycans, which contribute to the mechanical properties of the cervix (Feltovich & Carlson, 2017). The present study focuses on cervical epithelial cells as they play the central role in most of the biological processes that occur during CR, such as the maintenance of fluid balance, regulation of paracellular transport of solutes, mounting a protective and immunological barrier, acting as an “endocrine” gland, and differentiation (Mowa *et al*. 2008, Donnelly *et al*. 2013). Cervical epithelial cells also play a critical role in withstanding the gravitational pressure exerted by the growing fetus and maintaining the structural integrity of the cervix throughout cervical remodeling. Specifically, cervical epithelial cells provide immunological barrier defense via expression of toll-like receptors (TLRs), secretion of antimicrobials, cytokines, chemokines, and mucus (Mahendroo 2017, Xu *et al*. 2018).

Not only do cervical epithelial cells provide an immunological barrier, but also mounts a structural barrier directly by the cells themselves and intercellular junctions

(Reviewed by Barrios de Tomasi *et al*. 2019). Cervical epithelial cells are held together by intercellular junctions, which also regulate paracellular transportation and intercellular communication (Wira *et al*. 2005, Anton *et al.* 2017). One such junction located between cervical epithelial cells are tight junctions, which enable the epithelia to establish a physical barrier against pathogens as well as polarize the cells (Wira *et al*. 2014, Wessels *et al*. 2018). Estrogen regulates the expression and activation (via phosphorylation) of key intercellular junction proteins, such as occludin and claudin (Wira *et al*. 2015). Induction of occludin expression by estrogen can lead to short-term decrease in trans-epithelial resistance and subsequently in the reduction of the integrity of the physical barrier (Gorodeski 2007).

Our recent studies have also suggested that cervical epithelial cells are likely involved in withstanding the pressure of the growing fetus since they are the major source of mechano-sensitive signaling molecules during CR (Gordon & Mowa 2019). In part, the cervical epithelial cells withstand the gravitational force exerted by the growing fetus by proliferating and perhaps expressing regulatory signals that influence other cell types in the cervix, such as fibroblasts. It is well known that cervical epithelial cells undergo extensive proliferation and differentiation throughout pregnancy and CR. This cellular proliferation accounts for 50% of the entire growth of the cervical tissue in gestation (Nallasamy & Mahendroo, 2017). Specifically, epithelial cell proliferation is most pronounced during ripening of CR (Mahendroo *et al*. 1999, Mowa *et al*. 2008). In the rat model, increase in cervical wet weight is partially attributed to accumulation of new cells (proliferation) (Burger & Sherwood 1998). Lee *et al*. (2005) have shown that relaxin is one of the regulators of epithelial cell proliferation during cervical ripening and the second half of the rat pregnancy. The growth in cervical epithelial cell increases the circumference of the cervical lumen and thus the overall enlargement of the cervix (Lee *et al*. 2005), a process that is crucial for preparation of parturition. Furthermore, cervical epithelial proliferation is associated with increased mucus production, which provides immunological and mechanical protection, as well as anti-microbial function (Cunningham 2010). In the present study, we are specifically interested in investigating genome-wide gene expression of human cervical epithelial cell *in vitro* and their associated biological function. Based on our previous studies, it is likely that disruption of these biological functions in this cell type could impact CR and pregnancy and may likely lead to obstetric complications, including preterm labor. Therefore, we will also evaluate how these biological functions influence CR.

There are several regulatory factors that influence cervical epithelial cell proliferation during CR and they include estrogen, relaxin, and hyaluronan (Goldsmith *et al*. 1995, Lenhart *et al*. 2001, Word *et al*. 2007, Mahendroo 2017). Our lab has recently shown that vascular endothelial growth factor (VEGF) can also induce cervical epithelial cell proliferation (Mowa *et al*. 2008) and identified the genome-wide signature genes regulated by VEGF in the various cells of rat cervix during pregnancy. Because of the fundamental role of cervical epithelial cells in CR, we use isolated Human cervical epithelial cells in culture to characterize the genome-wide effect of VEGF on these cells.

VEGF is a member of a large family of growth factors that includes four isoforms of VEGF as well as placental growth factor (PIGF) (Conn *et al*. 1990, Tischer *et al*. 1991, Park *et al*. 1994, Shima *et al*. 1996, Ferrara & Davis-Smyth 1997). The isoforms include VEGF-A, -B, -C, -D, -E and PIGF. VEGF-A is the predominant and most studied isoform (Dulak *et al.* 2000, Zachary & Gliki 2001) and can be divided into several splice variants by the number of amino acids after signal sequence cleavage, namely 121, 165, 189 and 206 (Mueller *et al.*

2000). VEGFA-165 variant has been shown to be the most potent and abundant in humans (Zachary & Gliki 2001). There are three receptors known to mediate the biological properties of VEGF and they are: 1) fms-like tyrosine kinase-1 (flt-1) or VEGF receptor 1, 2) kinase domain receptor (KDR) or VEGF receptor 2, and 3) fms-related tyrosine kinase 4 (FLT4) or VEGF receptor 3 (de Vries *et al*. 1992, Mustonen & Alitalo 1995, Shibuya 1995, Ferrara & Davis-Smyth 1997, Shibuya *et al*. 1999). However, KDR and flt-1 are considered the primary receptors for vascular vessels, while FLT4 mainly mediates VEGF's effects in lymph vessels (Shibuya *et al*. 1999). VEGF is known to be a regulator of physiological and pathological angiogenesis. Such physiological processes include embryogenesis, skeletal growth and reproductive functions, while pathological processes involving VEGF-mediated angiogenesis include tumor formation and intraocular neovascular disorders (Shima *et al.* 1995, Ferrara *et al.* 2003). Previous studies have shown that induction of angiogenesis by VEGF is primarily mediated by the signaling molecule, protein kinase B (Six *et al*. 2002).

VEGF and its receptors have been found in the cervix of rodents (Mowa *et al*. 2004, Mowa *et al*. 2008). Previous DNA microarray studies in our lab have shown that blockage of production of local endogenous VEGF in the cervix of pregnant rats alters expression of approximately 4,200 genes, which are involved in various biological functions, namely cell proliferation, cell motility, circulation, tissue remodeling, immune response and heat shock protein activity (Mowa *et al*. 2008). These biological processes are closely associated with CR (Nguyen *et al*. 2012). Subsequent studies from our lab that followed the DNA microarray study showed that VEGF promotes cervical epithelial cell proliferation, trans-epithelial recruitment of WBC, and folding/edema (Mowa *et al*. 2008). Based on these earlier studies, we have proposed that VEGF's influence on cervical epithelial events likely play a crucial role in CR because 1) the cervix must grow to ensure the retention of the fetus in utero (proliferation), 2) vascular changes such as angiogenesis and vasodilation are necessary to ensure adequate supply of oxygen and nutrients to the cervix undergoing CR, and 3) VEGF plays a role in mediating a physiological inflammatory-like response in the cervix considered one of the hallmarks of CR.

Considering the various effects of VEGF on cervical events and the central role of cervical epithelial cells in CR, it is critical to profile the genome-wide expression of VEGF-regulated genes in cervical epithelial cells and categorize their biological functional groups. Therefore, the current study aims to delineate the signature genes regulated by VEGF in human cervical epithelial cells in an *in vitro* model.

Chapter Two: Experimental Design

Human cervical epithelial cells (CerEpiCells) were cultured *in vitro* and treated with exogenous VEGF, harvested and then analyzed using RNA sequencing, which was then verified by real-time PCR analysis.

Cell culture: CerEpiCells used in the present study were purchased from ScienCell Research Laboratories (Carlsbad, CA). Firstly, poly-L-lysine-coated T-75 flasks were prepared by mixing 10 mL of sterile water with 15 μL of poly-L-lysine stock solution [10 mg/mL] (ScienCell Research Laboratories, Cat. #0413 Carlsbad, CA), and then placed in a 37°C, 5% CO_2 incubator overnight under sterile conditions, according to the manufacturer's protocol. Complete basal medium (CerEpiCM, ScienCell Research Laboratories, Cat. #7061 Carlsbad, CA) was prepared by mixing 5 mL of Cervical Epithelial Cell Growth Supplement (CerEpiCGS, ScienCell Research Laboratories, Cat. # 7062 Carlsbad, CA) with 5 mL of penicillin/streptomycin solution (P/S, ScienCell Research Laboratories, Cat. # 0503 Carlsbad, CA), which were then aseptically added to 500 mL CerEpiCM (ScienCell Research Laboratories, Cat. #7061 Carlsbad, CA) under sterile conditions, per ScienCell Research protocol. After incubation, poly-L-lysine coated T-75 flasks were decontaminated using 70% ethanol, added to sterile field, rinsed twice with sterile water, and then 15 mL of complete medium was added to each flask. Cells were thawed from cryopreservation media in 37°C water bath and then added to each T-75 flask at a seeding density of 6,000 cells/cm^2. T-75 flasks containing cells were incubated overnight at 37°C and 5% CO_2. Culture media were replenished the following day to remove residual DMSO. Thereafter, media were changed every three days until the culture reached approximately 70% confluency and following that (reaching 70% confluency), media were changed every other day until the culture reached 90% confluency.

Sub-culturing cells: CerEpiCells were subcultured into new poly-L-lysine T-75 flasks after reaching 90% confluency, according to ScienCell Research Laboratories subculturing protocol. Prior to use the following solutions were thawed to room temperature: complete media, trypsin/EDTA solution (T/E, ScienCell Research Laboratories, Cat. # 0103 Carlsbad, CA), T/E neutralization solution (TNS, ScienCell Research Laboratories, Cat. #0113 Carlsbad, CA), Dulbecco's Phosphate-Buffered Saline (DPBS) (Ca^{++}- and Mg^{++}- free, ScienCell Research Laboratories, Cat. #0303 Carlsbad, CA) and fetal bovine serum (FBS, ScienCell Research Laboratories, Cat. #0500 Carlsbad, CA). Complete media were removed from cells and cells were then rinsed once with 10 mL DPBS. Following addition of 5 mL of DPBS and 5 mL of T/E solution to T-75 flasks containing cells, the flasks were incubated for 3 to 5 minutes, until cells were completely round in shape. During the incubation, a 50 mL conical centrifuge tube with 5 mL FBS (thawed) was prepared. T/E solution from T-75 flask was transferred to the 50 mL centrifuge tube containing FBS and the T-75 flask was incubated for an additional 1 to 2 minutes. At the end of the incubation, the flask was gently tapped to dislodge cells from the surface. Five mL of TNS solution was then added to the T-75 flask and detached cells were transferred to 50 mL centrifuge tube, followed by a second rinse of the flask with 5 mL TNS solution to collect residual cells. Cell harvest was considered successful if fewer than 5% of cells remained in the T-75 flask. The 50mL centrifuge tube containing harvested cells was then centrifuged at 1,000 rpm for 5 minutes.

The supernatant was removed, and cells were resuspended in 5 mL of CerEpiCM. Two 10 μL samples from the resuspended harvested cells were transferred to 1 mL Eppendorf tubes for cell count and viability calculations. Ten μL of Trypan Blue dye was added to each Eppendorf tube and mixed by pipetting and the stained cell suspension was transferred to the hematocytometer for cell count. Cells were counted in each quadrant and the average number of cells were determined by calculating the count in each 20 μL of resuspended cells in the Eppendorf tubes. The average cell count was then multiplied by 5,000 (constant), which was multiplied by 5 mL to determine cells/mL in the original cell suspension of the 50 mL centrifuge tube. Cells were then subcultured into new poly-L-lysine-treated T-75 flasks at a seeding density of 6,000 cells/cm^2. Cells were maintained as described above until growth reached 90% confluency or until treatment with VEGF.

VEGF Dose Optimization: Initial studies were conducted to determine the optimal dosage of exogenous VEGF on epithelial cells in culture. After sub-culturing CerEpiCells into 100 mm petri dishes (VWR, Cat. # 25373-100 Radnor, PA), cells were treated with three different dosages of exogenous recombinant human VEGFA-165 protein (Abcam, Cat. # Ab9571 Cambridge, MA) once cells had reached 70% confluency. First, 10 μg of VEGF protein was dissolved in 100 μL 0.1M PBS to constitute a stock solution at a concentration of 100 ng/μL, which was then stored at -20°C. Three replicates were used for each treatment group, as follows: **1**) Negative control (NC) – Vehicle (0.1M PBS) only with 0 ng/mL of VEGF treatment; **2**) VEGF dose-dependent treatments: **a**) medium, 30 ng/mL and **c**) high, 50 ng/mL. Specifically, the negative control group was treated with a mixture of the following: 0 μL of recombinant VEGF protein, 300 μL 0.1M PBS and 9.7 mL cervical epithelial cell medium. Cells in the 30 ng/mL treatment group were treated with 3 μL of stock recombinant VEGF protein [100 ng/μL], 297 μL 0.1M PBS, and 9.7 mL cervical epithelial cell medium and cells in the 50 ng/mL treatment group were treated with 5 μL of stock recombinant VEGF protein [100 ng/μL], 295 μL 0.1M PBS, and 9.7 mL cervical epithelial cell medium. After treatment with either vehicle only or recombinant VEGF protein, cells were incubated for 24 hours at 37°C and 5% CO_2. After the 24 hour culture, the cells were harvested to extract RNA, and real-time PCR was performed on three selected markers of proliferation and a VEGF-sensitive gene in order to determine the optimal dose of VEGF (Fig. 1). The real-time PCR protocols outlined below and described in our previous publications were utilized (Donnelly *et al*. 2013).

Time Optimization for VEGF Incubation: After optimizing the dosage for VEGF, we then conducted experiments to determine the optimal time for treating cells with VEGF under different dosages. Recombinant VEGFA-165 protein stock solution was prepared to a stock concentration of 10 μg/mL. Cells were plated in 100 mm petri dishes (VWR, Cat. 25373-100) at a seeding density of 6,000 cells/cm^2. Experiments with four treatment groups with 3 replicates each were conducted as follows: **1**) Negative control (NC)– NC group with vehicle only with no VEGF added, was harvested at 5 hours post treatment, **2**) VEGF treatment (optimization of incubation time and dose): **a**) Lower dose, short term: Cells were treated at a lower dose of 30 ng/mL of VEGF and then harvested at 5 hours post-treatment, **b**) High dose, short term: Cells were treated at a higher dose of 50 ng/mL of VEGF and harvested at 5 hours post-treatment, **c**) High dose, long term: Lastly, cells were treated at a higher dose of 50 ng/mL of VEGF and then harvested at 7 hours post-treatment. Specifically, in group 1

(NC) cells were grown in 15 mL cervical epithelial cell medium and RNA was extracted at 5 hours. In group 2a– cells treated with VEGF at a concentration of 30 ng/mL were grown in 15 mL cervical epithelial cell medium to which was added 45 μL recombinant VEGFA-165 [10 μg/mL] at time 0 and a booster of VEGF was added at 4 hours, after which cells were harvested and RNA was extracted at 5 hours post treatment to compare efficacy of the booster dose with VEGFA-165. In group 2b – cells treated with VEGF at a concentration of 50 ng/mL cells were grown in 15 mL cervical epithelial cell medium to which was added 75 μL recombinant VEGFA-165 [10 μg/mL] at time 0 and RNA extracted at 5 hours post-treatment. In group 2c – cells treated with VEGF at a concentration of 50 ng/mL cells were grown in 15 mL cervical epithelial cell medium to which was added 75 μL recombinant VEGFA-165 [10 μg/mL] at time 0, after which cells were harvested and RNA extracted at 7 hours post-treatment (Fig. 2). Real-time PCR was performed following the protocol outlined below and our previous publications, to determine optimal incubation time for treatment of cervical epithelial cells *in vitro* with exogenous recombinant VEGFA-165 protein.

RNA Extraction: RNA was extracted from cells using the Qiagen RNeasy Plus Mini Kit (Qiagen, Cat. # 74134 and 74136 Germantown, MD) for cell culture, as described below: Following the treatments described above, cells were harvested at 90% confluency by: **1**) first adding six hundred microliters Buffer RLT Plus to the petri dish containing cells; **2**) cells were then immediately scraped from culture dish, transferred to 1.5 mL Eppendorf tube and vortexed for 30 seconds: **3**) the homogenized lysate was then transferred to a gDNA Eliminator spin column placed in a 2 mL collection tube and centrifuged for 30 seconds at 10,000 RPM; **4**) the column was discarded, and the flow-through was saved; **5**) a volume of

600 μL 70% ethanol was added to the flow-through volume and mixed by pipetting; **6**) Seven hundred μL of flow-through sample were transferred to a RNeasy spin column placed in a 2 mL collection tube, centrifuged at 10,000 RPM for 15 seconds; **7**) the flow-through was then discarded, and steps 6 and 7 were repeated with the remainder of the sample; **8**) we then added 700 μL Buffer RW1 to the RNeasy Mini spin column placed in a 2 mL collection tube and centrifuged at 10,000 RPM for 15 seconds and discarded the flow-through thereafter; **9**) Next, 500 μL of Buffer RPE was added to RNeasy spin column, centrifuged at 10,000 RPM for 2 minutes, and the flow-through was discarded; **10**) Finally, RNeasy spin column was placed in a new 2 mL collection tube and centrifuged again for 1 minute at 10,000 RPM to further dry the membrane and **11**) lastly, the RNeasy spin column was placed in a new 1.5 mL collection tube and 30 μL RNase-free water was directly added to the spin column membrane and centrifuged at 10,000 RPM for 1 minute to elute the RNA trapped in the membrane.

<u>RNA Extraction Quality Analysis – Nanodrop</u>: After RNA extraction, 2 μL of the samples were placed on ice for spectrophotometry in order to determine the quality and quantity of the RNA samples using ThermoScientific Nanodrop 2000 (Waltham, MA), and the rest were snap-frozen and stored at -80°C. Two μL of elution water (RNase-free water) was loaded for "blanking" to initialize and standardize the equipment. RNA samples were then analyzed using 2 μL of each sample. The A260/A280 ratio, A260/A230 ratio, and the concentration of RNA (ng/μL) was recorded. The sample reader was cleaned using a KimWipe between each sample reading. RNase-free water was then added to each RNA sample to bring the final concentration of each sample to 200 ng/μL and mixed well by gently pipetting. Samples were then stored at -80°C until ready for use.

Reverse transcription: After RNA extraction, total RNA was converted into complementary DNA (cDNA) using reverse transcription that utilizes the enzyme reverse transcriptase (ThermoFisher Scientific, Cat. # 28025013 Waltham, MA). RT master mix was prepared according to the manufacturer's protocol and our previous studies per reaction: **a**) M-MLV Reverse Transcriptase Buffer (ThermoFisher Scientific, Cat. # 18057018 Waltham, MA) – 2 μL /tube, **b**) $MgCl_2$ (ThermoFisher Scientific, Cat. # AM9530G Waltham, MA) – 2 μL /tube, **c**) dNTP (ThermoFisher Scientific, Cat. # 10297018 Waltham, MA) – 2 μL/tube, **d**) RNase inhibitor (ThermoFisher Scientific, Cat. # AM2694 Waltham, MA) – 0.5 μL/tube, **e**) RT-PCR grade water (ThermoFisher Scientific, Cat. # AM9935 Waltham, MA) – 2 μL/tube, and **f**) Random Hexamer Primer (ThermoFisher Scientific, Cat. # SO142 Waltham, MA) – 1 μL/tube. Real-time PCR probes used for determining optimal incubation time, VEGF dose and CerEpiCell proliferation studies were purchased from AppliedBiosystem (Waltham, MA) and included: **1)** glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) (normalizer), **2)** minichromosome maintenance complex component 2 (*MCM2*), **3)** proliferating cell nuclear antigen (*PCNA*), **4)** marker of proliferation Ki-67 (*Ki-67*), and **5)** interleukin-6 (*IL-6*). Real-time PCR was also performed later to verify genomic data obtained from RNA sequencing (For details, see below). RT master mix was prepared and placed on ice or stored at -20°C. One microgram (5 μL) of RNA (200 ng/μL) from each sample were aliquoted into 0.2 mL PCR tubes. Total volume of RNA in these tubes was brought to 9.5 μL using RNase-free water and mixed gently by pipetting. Tubes were then incubated in a water bath set at 65°C for 5 minutes, followed by incubation at room temperature (23°C), while 9.5 μL RT master mix was added to each tube. Next, 1.0 μL of Reverse transcriptase was added to each tube, except for the DNA negative control tube. Tubes were then loaded into Thermocycler (Eppendorf Mastercycler – epGradient, Hamburg, Germany) and ran under the following conditions: 25°C for 10 minutes, 42°C for 2 hours, 95°C for 5 minutes and finally 4°C, at which the sample was left at until retrieved. cDNA was then stored at -20°C until used in RT-PCR analysis, described below.

Real-time PCR analysis: Gene expression analysis was used to optimize conditions for VEGF treatment of CerEpiCells in culture, as well as verify RNA seq results. Specifically, initial studies were conducted to optimize experimental conditions, including incubation time and VEGF dosage and later for verifying RNA sequencing data. In the initial optimization studies, a set of proliferative markers were used for optimization, including MCM-2, Ki-67, and PCNA. TaqMan Gene Expression Assays (ThermoFisher Scientific, Waltham, MA), which are pre-designed and pre-optimized gene-specific probe sets, were used and DNA amplification was performed using the Applied Biosystems real-time PCR machine (ABI 7300 HT) with the GeneAmp 7300 HT sequence detection system software (Perkin-Elmer Corp). The real-time PCR was set up in 96-well plates using a total volume of 25 μL per well. The reaction components included the following: **1**) 1000 ng (5.0 μL) of synthesized cDNA, **2**) 12.5 μL 2X Taqman Universal PCR Master Mix, **3**) 1.25 μL 20X Assays-on-Demand-Gene Mix (e.g. Ki-67), and **4**) 6.25 μL real-time PCR-grade RNAse-free water, and the program was set as follows: an initial step of 50 °C for 2 min and 95 °C for 10 min and then 40 cycles of 95 °C for 15 s and 60 °C for 60 s. The relative amount of the amplified genes was calculated from the threshold cycles with the instrument's software (SDS 2.0), according to the manufacturer's instructions. Relative expression levels of the target genes were normalized to the geometric mean of the endogenous control gene, *GAPDH*.

RNA-sequencing (NGS): Genome-wide experiments were conducted to identify VEGF-regulated signature genes in Human cervical epithelial cells, in order to have a comprehensive picture of VEGF's role in regulating these cells and its potential role in CR during pregnancy. After optimizing VEGF dosage, incubation time and cell culture conditions, cells were treated under these optimal conditions and were harvested, and total RNA was extracted and quantified. The extracted total RNA samples were then sent to Novogene Co., Ltd (Sacramento, CA) for RNA sequencing and bioinformatic analysis. Total RNA quantity and quality were assessed using Nanodrop (OD260/OD280). Agarose gel electrophoresis and Agilent 2100 (Bioanalyzer, Waldbronn, Germany) were used to assess RNA purity, integrity, and potential contamination. The library was then constructed, and quality assessed. Messenger RNA (mRNA) was purified from total RNA using poly-T oligo-attached magnetic beads. The mRNA was then fragmented randomly by addition of fragmentation buffer. The strand-specific library was then constructed by synthesizing the second strand cDNA using dUTP. Overhangs of purified double-stranded cDNA were converted into blunt ends, adenylation of 3' ends of DNA fragments, and NEBNext Adaptor with hairpin loop structure was ligated to prepare for hybridization and then the second strand of cDNA was digested by USER enzyme. The final library was then prepared by PCR amplification and purification of PCR products by AMPure XP beads. Bioinformatics analysis was then performed on raw data (Fig. 3).

Reads mapping to the reference genome: Reference genome and gene model annotation files were downloaded from genome website browser (NCBI/UCSC/Ensembl) directly (Novogene Co., Ltd.). Indexes of the reference genome was built using STAR and paired-end clean reads were aligned to the reference genome using STAR (v2.5) (Novogene Co., Ltd.). STAR used the method of Maximal Mappable Prefix (MMP) which generates a precise mapping result for junction reads (Novogene Co., Ltd.).

Quantification of gene expression level: HTSeq v0.6.1 was used to count the read numbers mapped of each gene and then fragments per kilobase of transcript per million mapped reads (FPKM) of each gene was calculated based on the length of the gene and reads count mapped to this gene. FPKM, Reads Per Kilobase of exon model per Million mapped reads, considers the effect of sequencing depth and gene length for the reads count at the same time, and is currently the most commonly used method for estimating gene expression levels (Mortazavi *et al*. 2008).

Differential expression analysis: Differential expression analysis between two conditions/groups (two biological replicates per condition) was performed using the DESeq2 R package (2_1.6.3). DESeq2 provide statistical routines for determining differential expression in digital gene expression data using a model based on the negative binomial distribution. The resulting P-values were adjusted using the Benjamini and Hochberg's approach for controlling the False Discovery Rate (FDR) (Benjamini & Yekutieli 2001). Genes with an adjusted P-value <0.1 found by DESeq2 were assigned as significantly differentially expressed.

RNA sequencing – Statistical Analysis: Downstream analysis was performed using a combination of programs including STAR, HTseq, Cufflink and wrapped scripts. Alignments were parsed using Tophat program and differential expressions were determined through DESeq2/EdgeR. Results that had adjusted p-value of 0.1 were considered statistically significant.

Real-time PCR – Statistical Analysis: Data for real-time PCR analyses were analyzed using Student's t-test. *P* values of <0.05 were considered to be statistically significant.

Chapter Three: Results

This chapter describes all the data obtained from the present study, including **a**) the preliminary data obtained during optimization experiments, **b**) RNA sequencing experiments and **c**) verification data of RNA sequencing by real-time PCR analysis.

Data from optimization studies

<u>Vascular endothelial growth factor (VEGF) upregulates expression of genes associated with proliferation in a dose-dependent manner:</u> CerEpiCells were treated with various dosages (30 ng/mL and 50 ng/mL) of human recombinant VEGFA-165 for 24 hours and gene expression levels were measured using real-time PCR (Fig. 4). Gene probes associated with proliferation (*MCM-2, Ki-67* and *PCNA*) were used to determine the optimal dose of VEGFA-165 to be 50 ng/mL (Fig. 4). The optimal dose (50 ng/mL) treated for 24 hours produced 125%-fold change in *MCM-2* expression as compared to cells receiving no treatment of VEGFA-165 (NC) (Fig. 4A). The 50 ng dose produced a 110% fold change in *PCNA* expression as compared to the negative control (Fig. 4B). *Ki-67* expression was down regulated by the 50 ng VEGF dose (Fig. 4C). Gene probe, interleukin-6 (*IL-6*) was used as a positive control to determine efficacy of treatment with VEGFA-165 protein (Fig. 4D) (Nguyen *et al*. 2012).

Cells were then treated with 50 ng/mL VEGFA-165 for 5 hours or 7 hours to determine optimal treatment time (Fig. 2). Cells were also treated with 30 ng/mL VEGFA-165 at time 0 and 4 hrs, harvested at 5 hours to compare efficacy of repeat dosing with VEGFA-165 (Fig. 2). Using real-time PCR and the same gene probes as discussed previously

(*MCM-2*, *Ki-67*, and *PCNA*), 7 hours was determined to be the optimal treatment time (Fig. 5). The optimal dose (50 ng/mL) treated for 7 hours produced 110%-fold change in *MCM-2* expression as compared to cells receiving no treatment of VEGFA-165 (NC) (Fig. 5A). The 50 ng dose produced a 111% fold change in *PCNA* expression as compared to the negative control (Fig. 5B). *Ki-67* expression was the most significantly up regulated gene by 50 ng VEGFA-165 treatment, with a 151%-fold change as compared to the negative control (Fig. 5C). Gene probe, interleukin-6 (*IL-6*) was used as a positive control again (Fig. 5D) (Nguyen *et al*. 2012).

Data from RNA sequencing experiments

<u>VEGF alters gene expression of a variety of genes in Human cervical epithelial cells associated with different biological themes:</u> When CerEpiCells were treated with optimized concentration of VEGF (50 ng/mL) in culture, it differentially altered the expression of 162 genes that are associated with a variety of biological functions or properties (Fig. 7, Table 1). The 162 genes were categorized into a total of nine biological groups, based on their primary biological properties. Genes were identified and described using Ensemble genome browser and HUGO Gene Nomenclature Committee (HGNC). The nine biological groups are: **1**) proliferation, **2**) energy metabolism, **3**) structure/matrix, **4**) immune response, **5**) cell adhesion/cell-cell communication, **6**) non-coding RNA, **7**) pseudogenes, **8**) uncharacterized and **9**) miscellaneous. Of these biological themes, the genes in only three groups showed significant differential expression, namely those associated with proliferation, energy metabolism, and extracellular matrix (Figs. 8, 9, and 10). These three biological groups had a total of twelve genes whose expression were found to be statistically significant with p-adjusted value (Padj) < 0.1. Data was analyzed and discussed below based on p-adjusted value of 0.1. Out of the twelve genes, **1**) Seven genes in CerEpiCells were found to be up-regulated by VEGFA-165 with fold changes ranging between 0.33 and 0.39 [Neural precursor cell expressed developmentally down-regulated protein 8 (*NEDD8*), FAU ubiquitin like and ribosomal protein S30 fusion (*FAU*), metastasis associated lung adenocarcinoma (*MALAT1*), mitochondrially encoded ATP synthase 6 pseudogene 1 (*MTATP6P1*), mitochondrially encoded cytochrome c oxidase III (*MT-CO3*), mitochondrially encoded ATP synthase 6 (*MT-ATP6*), mitochondrially encoded cytochrome c oxidase subunit 2 (*MT-CO2*)] (Fig. 8 and 9); and **2**) Five genes were found to be down-regulated with fold changes between -0.44 and -0.39 [solute carrier family 6 member 14 (*SLC6A14*), collagen type 1 alpha 1 chain (*COL1A1*), C-X-C motif chemokine ligand 14 (*CXCL14*), sestrin 3 (*SESN3*), keratin 4 (*KRT4*)] (Figs. 8 and 10).

VEGF differentially alters expression of genes associated with proliferation in Human cervical epithelial cells: Exogenous VEGFA-165 was found to differentially alter the expression of a total of nineteen genes associated with proliferation, out of the 162 genes that were differentially expressed, i.e., proliferation genes constituted 12% of the total altered genes (Table 2). Of the nineteen proliferation associated genes, the majority of these genes (9, i.e., about 47%) were down-regulated, with the rest (10, i.e., 53%) up-regulated (Table 2). Further, out of nineteen genes, only six genes were significantly differentially expressed, of which half (*NEDD8*, *FAU*, and *MALAT1*) were up-regulated (*NEDD8*, *FAU*, and *MALAT1*) and the remaining half were down regulated (*CXLC14, SESN3, SLC6A14*) (Padj < 0.1) when CerEpiCells were treated with VEGFA-165 (Table 2, Fig. 8). The log2 fold changes for

NEDD8, FAU, and *MALAT1* were 0.33, 0.35, 0.33, respectively and that of *CXCL14*, *SESN3* and *SLC6A14* were -0.42, -0.42, -0.44, respectively (Table 2). To verify these RNA seq (next generation sequence) data showing VEGF's effects on the expression pattern of proliferative genes, real-time PCR was used on the following select genes (*SESN3* & *MT-ATP6*) (Figs. 11 and 12).

VEGF differentially alters expression of genes associated with energy metabolism and Matrix in Human cervical epithelial cells: As stated previously, among the biological groups with genes that were significantly expressed included those associated with extracellular matrix, mitochondrial function and proliferation. Of the 162 differentially expressed genes, VEGF was found to regulate seven genes categorized as energy metabolism. Four of the twelve significantly differentially expressed genes were categorized as energy metabolism and were significantly up-regulated in cells treated with VEGFA-165 as compared to the negative control. These included *MTATP6P1*, *MT-CO3, MT-ATP6, MT-CO2* (Fig. 9). Specifically, the fold change of *MTATP6P1, MT-CO3, MT-ATP6, MT-CO2* were 0.39, 0.34, 0.39, 0.39, respectively. Two genes associated with extracellular matrix were significantly down-regulated (*COL1A*, *KRT4*) in cells treated with VEGF as compared to the negative control (Fig. 10). The fold changes for these two genes were -0.41 (*COL1A1*) and -0.39 (*KRT4*).

Verification of RNA sequencing data by real-time PCR analysis

RNA-seq results of select genes (*SESN3* & *MT-ATP6*) were verified using real-time PCR. The gene expression using these two gene probes (*SESN3* & *MT-ATP6*) was measured using cells treated with 50ng exogenous VEGF for 7 hours compared to cells receiving no VEGF treatment (NC) following the Reverse Transcription protocol as described previously. *GAPDH* was used as a normalizer. Real-time PCR analysis showed that *SESN3* is significantly down regulated by exogenous VEGF in CerEpiCells, whereas *MT-ATP6* is up regulated (Fig. 11, p-value < 0.05). These results match RNA-seq data.

Chapter Four: Discussion

Cervical epithelial cells have been shown to play a central role in reproductive events, notably cervical remodeling and, consequently, their dysfunction could possibly be implicated in PTB (Laurent & Fraser 1992, El Maradny *et al*. 1997). We have previously delineated VEGF-regulated genes and biological functions in the different cell types of rat cervix during pregnancy using VEGF inhibitors and DNA microarray (Mowa *et al*. 2008). Specifically, based on these earlier studies, VEGF's biological effects on all the cervical tissue cell types of pregnant rats include proliferation, immune response, tissue remodeling, cell motility, circulation, and heat shock protein activity (Mowa *et al*. 2008). The biological themes of some of these DNA microarray data were later confirmed by our most recent studies that showed that local administration of exogenous recombinant VEGF in mouse cervix induces marked proliferation and growth of epithelial cells. We also demonstrated increased intercellular permeability and immune cell infiltration into cervical lumen (Donnelly *et al*. 2013). Therefore, the present study focused on teasing out the specific VEGF-regulated signature genes in human cervical epithelial *in vitro* using RNA seq. Of the total 25,000 + genes that were examined in human cervical epithelial cells, 162 genes were found to be differentially expressed (Fig. 7). These 162 genes were then categorized into nine different biological groups, namely: 1) proliferation, 2) immune response, 3) structure/matrix, 4) mitochondrial function, 5) cell adhesion/communication, 6) pseudogenes, 7) non-coding RNA, 8) miscellaneous genes and 9) uncharacterized genes (Table 1), consistent with earlier findings from rodent studies. Real-time PCR was used to verify RNA seq data. Finally, out of the total 162 genes that were differentially regulated by VEGFA-165, only twelve were found to be statistically *significantly* expressed based on p-adj value <

0.1. The twelve genes were further characterized into three functional biological groups, namely proliferation, energy metabolism and matrix (Figs. 8, 9, and 10). It is important to note that some genes are associated with multiple functions, and, therefore, belong to more than one category.

Consistent with findings of our earlier rodent studies, the present study found that VEGF altered the gene expression of proliferative genes in human cervical epithelial cells the most (19/162) and out of the 12 genes that were significantly differentially expressed, six belonged to proliferative genes (*CXCL14, SESN3, SLC6A14, NEDD8, FAU, MALAT1*). However, there was a difference in the specific types of proliferative genes involved and the pattern of expression, likely reflecting species differences as well as the type of models used, i.e., *in vitro* (Human) versus *in vivo* (Rodents). While VEGF mostly promoted expression of proliferation genes in human cervical epithelial cells, it down regulated expression of some of the proliferation genes and those that inhibited proliferation. For example, *SESN*3, which inhibits proliferation, was down regulated by VEGF. Also, as discussed earlier, some of the genes associated with proliferation also exerted other biological effects, such as *CXCL14*, a chemokine primarily involved in immune response but also inhibits proliferation of breast cancer and endothelial cells (Shellenberger *et al.* 2004, Noonan *et al.* 2008, Gu *et al.* 2012).

VEGF was found to down regulate expression of *CXCL14* in human cervical epithelial cells in the present study (Fig. 8). *CXCL14*, which has previously been shown to be localized in the cervix (Frederick *et al*. 2000, Lu *et al*. 2016), encodes for a homeostatic chemokine that belongs to a superfamily of small chemotactic cytokines (Hernández-Ruiz & Zlotnik 2017). *CXCL14* regulates multiple functions, including cell survival, angiogenesis, tumor cell proliferation and immune response (Rollins 2006, Zlotnik 2006). Of interest to the present study, *CXCL14* has significant expression in mucosa with three other chemokines (CCL28, CCL25, CXCL17). These four chemokines also have broad anti-microbial activity, suggesting their potential to influence the composition of the mucosal microbiome. Specifically, CXCL14 exhibits antimicrobial activity against Gram-negative *Escherichia coli* (*E. coli*), Gram- positive *Staphylococci* species, *Propionibacteria, Pseudomonas aeruginosa, Streptococcus* species, and the yeast *C. albican* (Dai *et al*. 2015). *CXCL14* could, therefore play a relevant role in local cervical immune surveillance (Maerki *et al.* 2009, Dai *et al*. 2015). It is however not clear for now why VEGF down regulates CXCL14 since we have previously shown that VEGF induced immune response in pregnant rats and specifically promoted immune recruitment into cervical lumen of non-pregnant mice cervix (Donnelly *et al*. 2013). However, we did not specifically investigate the immune cell types that were recruited by VEGF. Since *CXCL14* does not recruit all immune cell types (B-cells, monocytes, neutrophils, and dendritic cells) (Cao *et al*. 2000, Sleeman *et al*. 2000, Kurth *et al*. 2001), it is likely that it (*CXCL14*) does not mediate VEGF-induced immune recruitment in the cervix (Donnelly *et al*. 2013, Stanley *et al*. 2018). Perhaps another reason VEGF diminished levels of *CXCL14* in cervical epithelial is because *CXCL14* appears to also oppose another key role of VEGF, namely angiogenesis. *CXCL14* enhances maturation of dendritic cell (DC) (Shellenberger *et al.* 2004, Noonan *et al.* 2008), which (maturation of DC) has been found to be inhibited by VEGF (Sozzanni *et al.* 2007). We have previously shown that VEGF promotes angiogenesis in the cervix during pregnancy and proposed that this biological property of VEGF plays a critical role in CR (Mowa *et al*. 2004). *CXCL14* has also been found to inhibit proliferation in breast cancer cells *in vitro* (Gu *et al.* 2012). Taken together, we reason that VEGF down-regulates *CXCL14* in an effort to promote cervical epithelial cellular proliferation, which *CXCL14* likely inhibits. The present findings confirm *CXCL14*'s role in mucosa and that VEGF may attenuate *CXCL14*'s immune and anti-proliferative effects in cervical mucosa. However, the exact role of *CXCL14* in cervical events and its mode of interaction with VEGF requires more study. Future studies should investigate *CXCL14*'s specific role in the immune response found in cervical mucosa as well as determine the specific VEGF-receptor and subsequent signaling pathways used to alter expression of *CXCL14* by VEGF.

SESN3 was also found to be down-regulated by the present study in human cervical epithelial cells after treatment with recombinant VEGFA-165. *SESN3* belongs to the sestrin family, which is a highly conserved family of stress-responsive proteins whose expression is primarily regulated via p53 signaling pathway (SESN1 and SESN2) (Nogueira *et al.* 2008, Budanov *et al*. 2010). Different from the other two sestrin molecules, SESN3 is up-regulated by forkhead box transcription factor belonging to O-subclass (FoxO), a downstream effector molecule from Akt (Nogueira *et al.* 2008). FoxO activity is negatively regulated by Ras signaling through the AKT/protein kinase B (PKB) and ERK protein kinases (Yang & Hung 2009). Therefore, activation of the Ras signaling pathway results in down-regulation of *SESN3* (Kopnin *et al.* 2007, Budanov *et al.* 2010). Although the underlying mechanism that VEGF may use to down regulate levels of *SESN3* in human cervical epithelial cells as revealed by the present study are for now unclear, it is interesting to note that the molecules of the Ras pathway (AKT/protein kinase B (PKB) and ERK protein kinases), which down regulate *SESN3*, are established key signaling molecules used by VEGF and cancer cell proliferation and endothelial cells (Zhong *et al.* 2000, Fang *et al.* 2007, Claesson-Welsh

2016). It is possible that this negative regulation of *SESN3* by VEGF may occur due to the down regulation of FoxO activity by Ras activation (Yang & Hung 2009). SESN3 is positively regulated by FoxO transcription factors (Nogueira *et al*. 2008), therefore when Ras signaling is activated by VEGF (Cross & Claesson-Welsh 2001), it in turn likely down-regulates FoxO signaling and thus *SESN3*. The down regulation of *SESN3* was confirmed by RT-PCR analysis, which showed *SESN3* expression is significantly (p-value < 0.05) less in VEGF treated cells as compared to *SESN3* expression in NC.

The third gene down-regulated by VEGF (Fig. 8), *SLC6A14*, is a transporter of multiple amino acids and therefore plays a critical role in the maintenance of amino acid nutrition (Babu *et al.* 2015). Of interest to the present study, among the amino acids SLC6A14 transports is arginine, a crucial ingredient for nitric oxide (NO) production (Gupta *et al.* 2006), an inducer of VEGF expression. NO also enhances the activity of hypoxia inducible factor (HIF), one of the most potent transcription factor of *VEGF* (Kimura & Esumi 2003). Based on these facts, one would have therefore thought that VEGF would up regulate *SLC6A14* so that there would be more raw material for NO, arginine, transported into cervical epithelial cells to promote activity of HIF, which would then up regulate VEGF to stimulate epithelial proliferation. This speculation is in line with the fact that blockage of *SLC6A14* inhibits amino acid transport and subsequently cancer growth (Gupta *et al.* 2006). It appears that *SLC6A14* may not play a significant role in proliferation of cells under physiological conditions since its level of expression is normally very low, including in cervical epithelial cells (Gupta *et al.* 2006). The results shown by Gupta *et al.* (2006) match the findings of the present study. More studies are required to tease out the intricate relationship between *SLC6A14*, NO, HIF and VEGF in cervical epithelial cells under different physiological conditions and how this relates to CR and preterm birth.

We also found that VEGF upregulate levels of *NEDD8* in human cervical epithelial cells *in vitro* by the present study (Fig. 8). *NEDD8* is a regulatory protein involved in a range of fundamentally key biological activities in cell growth, viability and development (Mori *et al*. 2005, Ryu *et al*. 2011). It exerts its effects by activating a class of scaffold proteins, cullin-RING ubiquitin ligases (CRLs) (Ryu *et al*. 2011), which comprise the largest family of E3 enzymes involved in the ubiquitin-proteasome system (UPS) (Duda *et al*. 2008, Deshais 2017). The UPS uses enzyme-mediated specificity to regulate and degrade specific proteins (Varshavsky 2005). Each type of CRL associates with different combinations of proteins to form a multiprotein complex composed of an adaptor protein, substrate receptor and the target substrate (Scott *et al*. 2016). It is most likely that the *NEDD8* up regulated by VEGF in the present study activates CRL-2, whose adaptor protein and substrate receptors are EI-C and SOCS-box, respectively, and are associated with the Von Hippel-Lindau protein (VHL). The target protein of this multiprotein complex is HIF (Kershaw & Babon 2015), a transcription factor that regulates expression of more than 100 genes, including cell proliferation, apoptosis, angiogenesis, glycolysis, iron metabolism and others, under low oxygen conditions as reviewed by Schofield and Ratcliff (2004). Under hypoxic conditions HIF-α is not degraded (Schofield & Ratcliffe 2004, Ryu *et al*. 2011). However, when oxygen levels are adequate the 402 and 564 proline residues of HIF-α are hydroxylated by prolyl hydroxylase (PHD 2/3), which then becomes detectable to VHL and is subsequently presented for degradation by the 26S proteasome (Schofield & Ratcliffe 2004). In summary, when VEGF up regulates *NEDD8* in human cervical epithelial cells, as revealed by the present study, it leads to degradation of its transcription factor, HIF-α, which in turn will mitigate VEGF expression and its downstream effects. Therefore, we propose that VEGF in cervical epithelial cells appear to exert a negative feedback effect on HIF-α *in vitro*. It is not clear whether this is the same under *in vivo* conditions or is not true for cervical epithelia cells since we have shown previously that levels of VEGF increase over the course of pregnancy and decrease after birth in rodents (Mowa *et al*. 2004, Donnelly *et al*. 2013, Stanley *et al*. 2018). More studies need to be conducted to verify these hypotheses.

FAU was also up-regulated by VEGF (Fig. 8); however, little is known about their exact interactions. *FAU* is considered a house-keeping gene that regulates apoptosis of epithelial and T-cell lines and also possesses immunomodulatory and anti-microbial activities (Pickard 2012). *FAU* expression in normal tissues is largely invariant, however its expression has been found to be down-regulated in a number of human cancers (Pickard 2012). The up-regulation of *FAU*'s pro-apoptotic function is in contrast to the overall function of VEGF (angiogenesis and proliferation). Perhaps overexpression of VEGF indicates an aberrant tissue growth and may trigger *FAU* expression. However, further research is required to fully understand VEGF – *FAU* interactions.

The sixth gene characterized as having proliferative function is *MALAT1,* which is also up-regulated by VEGF (Fig. 8). *MALAT1* encodes a highly conserved nuclear non-coding RNA that promotes vascular formation and plays an important role in tumor-driven angiogenesis (Gutschner *et al.* 2012). Similar to the up-regulation of *MALAT1* induced by VEGF in cervical epithelial cells, Li *et al.* (2017) found a positive correlation between *MALAT1* and VEGF expression in both umbilical cord cells and mesenchymal stem cells.

More specifically, VEGF mRNA and protein expression were increased after overexpressing *MALAT1* in cells derived from patients with pre-eclampsia (Li *et al.* 2017), suggesting a reciprocal relationship between VEGF and *MALAT1*. Interestingly, pre-eclampsia is associated with maternal complement activation (Derzsy *et al.* 2010), and Gonzalez *et al.* (2011) has shown that complement activation in the mouse model is also linked to CR and pre-term birth, which indicates VEGF and *MALAT1* could play a crucial role in CR.

Of the twelve significantly differentially expressed genes, four genes associated with mitochondrial function and ATP synthesis were found to be significantly up-regulated by VEGF in human cervical epithelial cells in the present study (*MT-ATP6P1, MT-CO3, MT-ATP6, MT-CO2*) (Fig. 9). *MT-ATP6P1* is a pseudogene or a segment of DNA related to the original gene (*MT-ATP6*) which may or may not have lost some functionality relative to the complete gene (Tutar 2012). Tutar (2012) reports that pseudogenes can also perform regulatory functions. *MT-ATP6P1* has been shown to encode for accessory proteins of vacuolar (H+) – ATPases (V-ATPases) (Forgac 2007). V-ATPases combine energy from ATP hydrolysis to proton transport across membranes of eukaryotic cells (Forgac 2007). In a study by Pareja *et al*. (2018) in human embryonic cells when loss of function mutation occurs in *MT-ATP6P1* it results in decreased V-ATPase activity as well as decreased endosomal acidification. Endosomal acidification is also important for homeostasis and maturation of endosomes which are transport vesicles found in the cytoplasm (Hu *et al*. 2015). *MT-ATP6* encodes a subunit of ATP-synthase, the final step in oxidative phosphorylation where ATP is produced (Genetics Home Reference – MTATP6, Weber & Senior 2004). Real-time PCR analysis showed that VEGF up regulates *MT-ATP6* as compared to NC, however the expression levels were not significantly different. Little is known about the interaction of *MT-ATP6P1* or *MT-ATP6* specifically in cervical epithelial cells or in relation to VEGF.

MT-CO2 and *MT-CO3* are closely linked genes which encode for cytochrome C oxidase (COX) subunits two and three, respectively (Genetics Home Reference – MTCO2, Genetics Home Reference – MT-CO3). Both genes are functionally active in respiratory electron transport, ATP synthesis and heat production (Gene Cards). COX is the last enzyme in the electron transport chain that powers ATP synthase to produce ATP (Li *et al*. 2006). No studies to date have shown a direct interaction between VEGF and *MT-CO2* or *MT-CO3* in human cervical epithelial cells. In mouse fibroblast cells, Li *et al*. (2006) found that when COX is dysfunctional it leads to compromised mitochondrial membrane potential, decreased ATP production, and decreased growth of cells in media; thus, indirectly linking the importance of COX in cell growth and proliferation. However, the direct relationship between COX and VEGF remains to be elucidated, specifically in cervical epithelial cells.

The findings of our present study on VEGF’s effect on expression of genes related to mitochondrial function are critical as they extend VEGF’s impact on cervical epithelial physiology beyond its traditionally established roles to date. They (findings) imply that VEGF does not only ensure adequate oxygen perfusion of cervical tissue via angiogenesis, as well as proliferation of cervical epithelial cells, but it (VEGF) also stimulates production of the energy required to power these energy-dependent processes. Therefore, local dysfunction of VEGF synthesis will consequently have much broader implications beyond just disrupting angiogenesis; dysfunctional VEGF could potentially threaten to shut down cervical epithelial function altogether. Although these findings are the first to be reported in cervical epithelial cells, they are consistent with several earlier studies conducted in endothelial cells from different tissues (Guo *et al.* 2017). For instance, a study by Guo *et al.* (2017) showed that VEGF treatment of human umbilical vein endothelial cells (HUVECs) increases mitochondrial oxidative respiration and intracellular ATP levels. According to this study (Guo *et al.* 2017), VEGF promoted mitochondrial function in HUVECs through the mammalian target of rapamycin (mTOR) signaling pathway. Mitochondrial function can also be induced upstream of VEGF by hypoxia and HIF. Specifically, hypoxia induces mitochondrial generation of reactive oxygen species (mROS) (Chandel *et al.* 1998). In turn, mROS will then stabilize VEGF's transcription factor, HIF subunits (Chandel *et al.* 2000, Kimura & Esumi 2003), by blocking the enzyme, propyl hydroxylase, that tags HIF for VHL to trigger ubiquitination and subsequent degradation (Ryu *et al*. 2011). Of particular interest to the present study, others have also shown that mitochondrial ROS are crucial to the regulation of cell proliferation (Sena & Chandel 2012). It is therefore likely that unlike the *NEDD8* relationship discussed earlier, a positive feedback loop between VEGF and HIF (and perhaps cervical epithelial proliferation) exists; i.e. VEGF up regulates expression of mitochondrial genes, which then promotes production of ATP to power cervical epithelial events such as proliferation and also generates mROS leading to the stability of HIF. HIF in turn up regulates expression of VEGF which then regulates and powers several cervical epithelial events. In addition, a review by Hamanaka and Chandel (2010) states that mitochondrial generation of ROS is also necessary for other essential biological signaling events, such as transcription, calcium storage, and energy storage. Other studies have also shown that VEGF induces mitochondrial biogenesis (Wright *et al.* 2008). More mechanistic studies need to be conducted in cervical epithelial cells to test these speculations. Also, because of the importance of energy metabolism to cell survival, there are likely multiple and redundant regulatory factors involved in cervical epithelial cell energy metabolism.

Two genes (*KRT4, COL1A1*) classified as extracellular matrix genes were both down-regulated in cervical epithelial cells treated with VEGF (Fig. 10). Consistent with earlier findings from our lab's *in vivo* model with rodents, VEGF alters the gene expression of some matrix genes, mainly *COL1A1*. On the other hand, while *KRT4* was found to be altered by VEGF *in vitro* in the current study, the microarray data from our previous studies did not show altered expression of this gene *in vivo* using the rodent model. The cytoskeleton (*KRT4*) plays a crucial role in cell cycle progression, cell death, and differentiation (Ahn *et al*. 2004) and is therefore a critical structure to consider in regard to cellular growth and proliferation.

COL1A1 is a gene which encodes for collagen, a cellular structure protein (Ahn *et al.* 2004) and under pathological conditions is typically associated with metastasis (Chen *et al.* 2003). Furthermore, *COL1A1* is concomitantly up-regulated with increased VEGF expression in tumors (Calvo *et al.* 2008). This is in direct contrast to our findings, which showed that *COL1A1* is down-regulated upon treatment with VEGF (Fig. 10). Perhaps, as Chen *et al.* (2003) suggests, there is a complex and coordinated mechanism of gene regulation occurring between the cervical epithelium and the microenvironment (i.e stromal cells, fibroblasts, etc.), specifically in cancer progression. Therefore, based on this logic we propose that the down-regulation of *COL1A1* upon VEGF treatment is due to the lack of interaction between cervical epithelial cells and the other cervical cellular type (i.e. stromal cells), since we are using an *in vitro* model of cervical epithelial cells. It is also possible that

COL1A1 expression is regulated differently in different cell types (i.e cervical epithelial cells versus fibroblasts).

KRT4 encodes a keratin and plays a protective role from mechanical and non-mechanical stress in epithelial cell function, which could lead to apoptosis (Coulombe & Bishr Omary 2002). While *KRT4* is not well characterized in healthy cervical epithelial cells, Wong *et al.* (2006) showed that *KRT4* is down-regulated in cervical squamous cell carcinoma and while, simultaneously, VEGF is up-regulated. These results are consistent with our present findings showing down-regulation of *KRT4* by VEGF. VEGF is known to play a crucial pathological role in tumor angiogenesis and proliferation (Gaffney *et al.* 2003). Therefore, perhaps VEGF negatively regulates apoptosis of cells via *KRT4* in order to induce proliferation of cells.

The sex steroid hormones, estrogen and progesterone, play the central role in female reproductive events during menstrual cycle and pregnancy (e.g., CR). Similar to VEGF, estradiol promotes epithelial cell proliferation while, in contrast, progesterone inhibits proliferation of these cells (Chung 2015, Mehta *et al.* 2016). Of interest to the present findings, multiple studies have demonstrated that estradiol increases levels of VEGF in multiple cell types (Cullinan-Bove & Koos 1993, Shifren *et al*. 1996, Bausero *et al*. 1998, Hyder *et al.* 2000, Mueller *et al*. 2000, Soares *et al.* 2002, Soares *et al.* 2004) and during CR, the two sex steroids (Estrogen and progesterone) regulate most of the genes expressed by the epithelial tissue (Andersson *et al.* 2008, Timmons *et al.* 2010). In fact, Havelock *et al.* (2005) reports that expression of estrogen- and progesterone-sensitive genes are more pronounced in the cervix compared to uterine fundus. Importantly, we have previously demonstrated that

VEGF and it's signaling molecules are present in the cervix of non-pregnant women and up regulated over the course of pregnancy as levels of sex steroid hormones increase and sharply decrease immediately after birth (Mowa *et al*. 2004, Donnelly *et al*. 2013). Furthermore, we have shown that exogenous estrogen up regulates expression of VEGF in mice cervix (Ohashi *et al*. 2014) implying that estrogen may either act up stream of VEGF or directly regulate the expression of VEGF-regulated genes, revealed in the present study. Also, several studies have shown that estrogen alters expression of several of these genes in different tissues. Of the twelve differentially expressed genes, nine are sensitive to estrogen. Seven of these genes are up-regulated by estrogen in various cell types (*CXCL14, SESN3, FAU, MALAT1, MT-CO3, COL1A1*) (Ivanova *et al*. 2013, Markiewicz *et al*. 2013, McCracken & Eldridge 2015, Ren *et al*. 2015, Sjöberg *et al.* 2016, Klinge 2018), while only one gene is down-regulated (*KRT4*) (Walker *et al*. 2007). Interestingly and opposite to estrogen regulation, *NEDD8* was found to be a regulator of ER-α expression in breast cancer tissue (Jia *et al*. 2019). There is no information to date on the relationship of estrogen and the remaining two genes, *MT-ATP6P1* and *MT-ATP6*. The implications of the present findings to CR and their underlying mechanism are currently unclear and thus will require more studies in the future. Future studies should determine the interaction and relationship between estrogen, VEGF, and the aforementioned genes in cervical epithelial cells.

In summary, the present study has shown that VEGF induces expression of multiple genes in CerEpiCells *in vitro* (Fig. 12). Particularly, the majority of the genes that VEGF induces in CerEpiCells are those associated with proliferation, a biological process which plays a crucial role in CR. Collectively, these findings suggest that VEGF plays a key role in CR by inducing expression of several genes that regulate three biological functions that are necessary for cervical remodeling to occur normally (cell proliferation, mitochondrial function, and cell structure integrity).

Figures and Tables

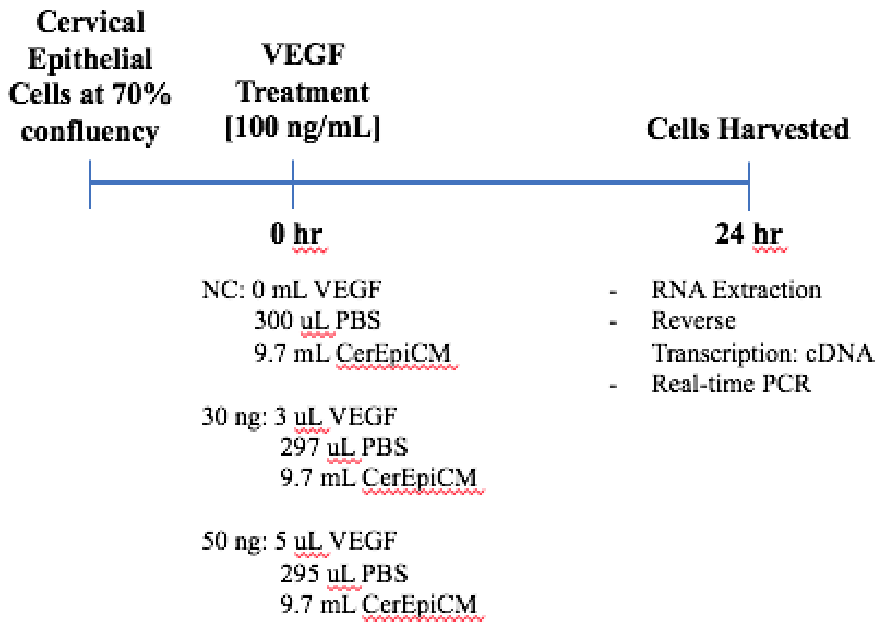

Figure 1: Schematic diagram of VEGF dose optimization experiments.

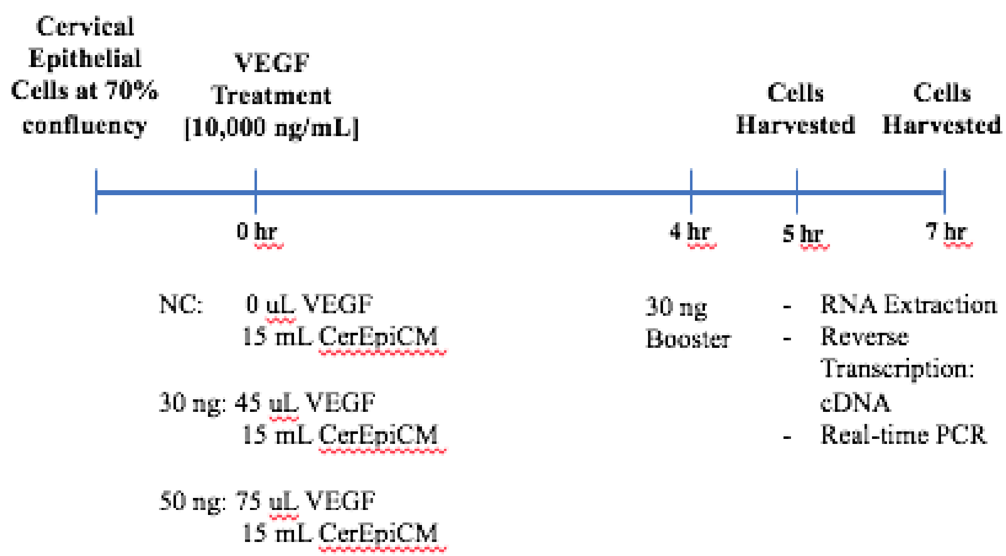

Figure 2: Schematic diagram of VEGF incubation time optimization experiments.

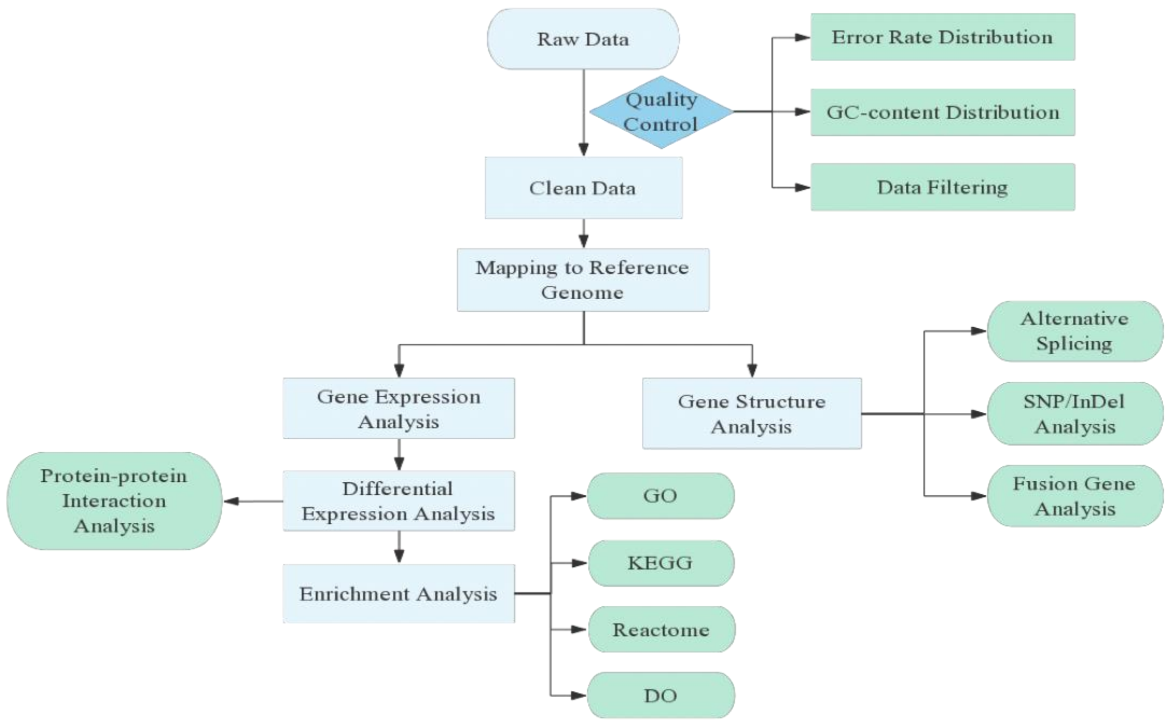

Figure 3: Workflow map of RNA sequencing bioinformatic analysis

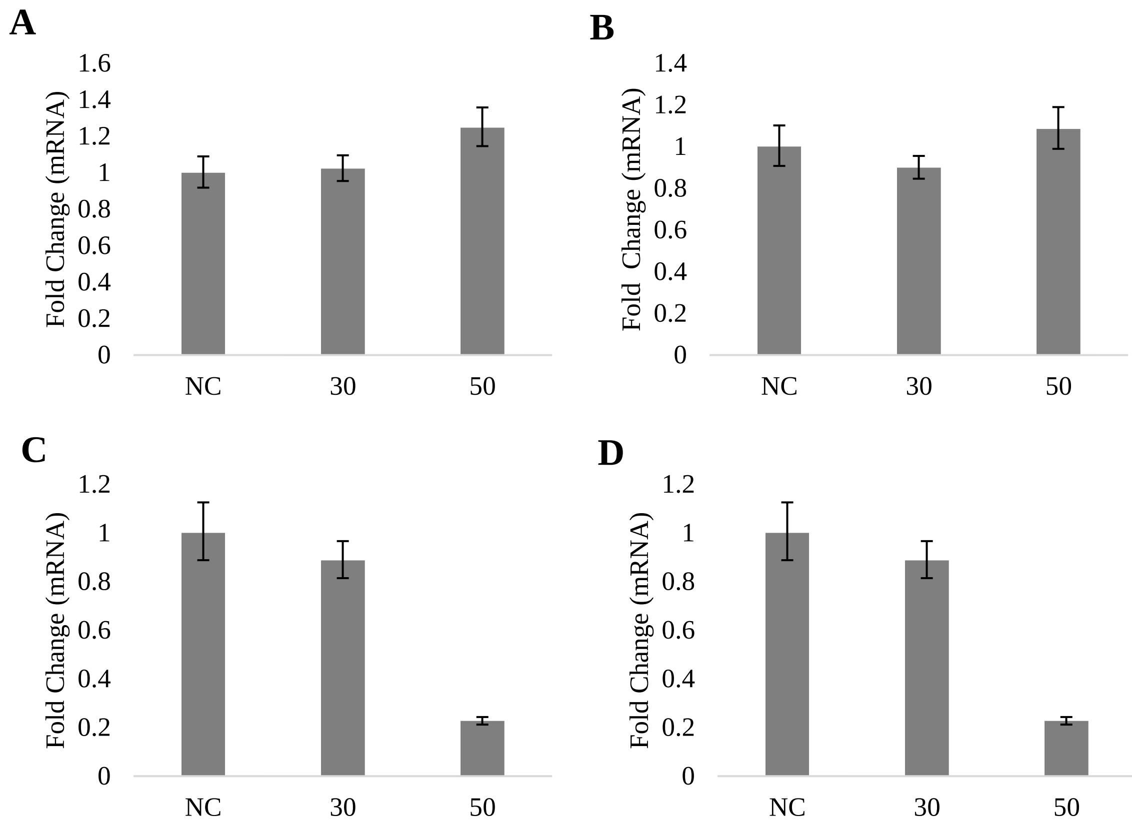

Figure 4. Human recombinant vascular endothelial growth factor – 165A protein (VEGFA-165) upregulates gene expression of genes associated with proliferation in a dose-dependent manner. Levels of MCM-2 (**A**) and PCNA (**B**) mRNA in Human cervical epithelial cells were up-regulated in cells treated with 50 ng/mL VEGFA-165 protein for 24 hours, as revealed by real-time PCR. Levels of Ki-67 (**C**) were not significantly increased by either 30 ng/mL or 50 ng/mL VEGFA-165 protein. IL-6 probe was used as a positive control (**D**) (n=4).

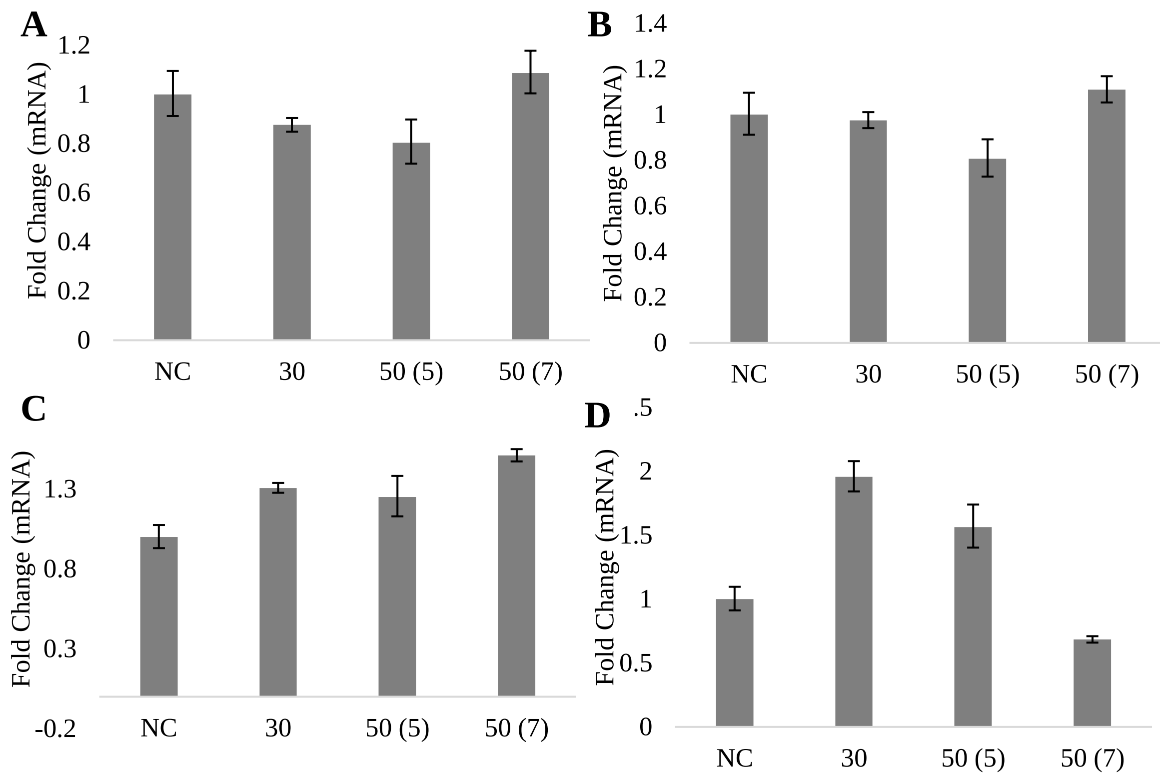

Figure 5. Human recombinant vascular endothelial growth factor protein (VEGFA-165) upregulates gene expression of genes associated with proliferation. Levels of MCM-2 (**A**), PCNA (**B**), and Ki-67 (**C**) mRNA in Human cervical epithelial cells were up-regulated with cells treated with 50 ng/mL VEGFA-165 protein for 7 hours, as revealed by real-time PCR. IL-6 probe was used as a positive control (**D**) (n=4)

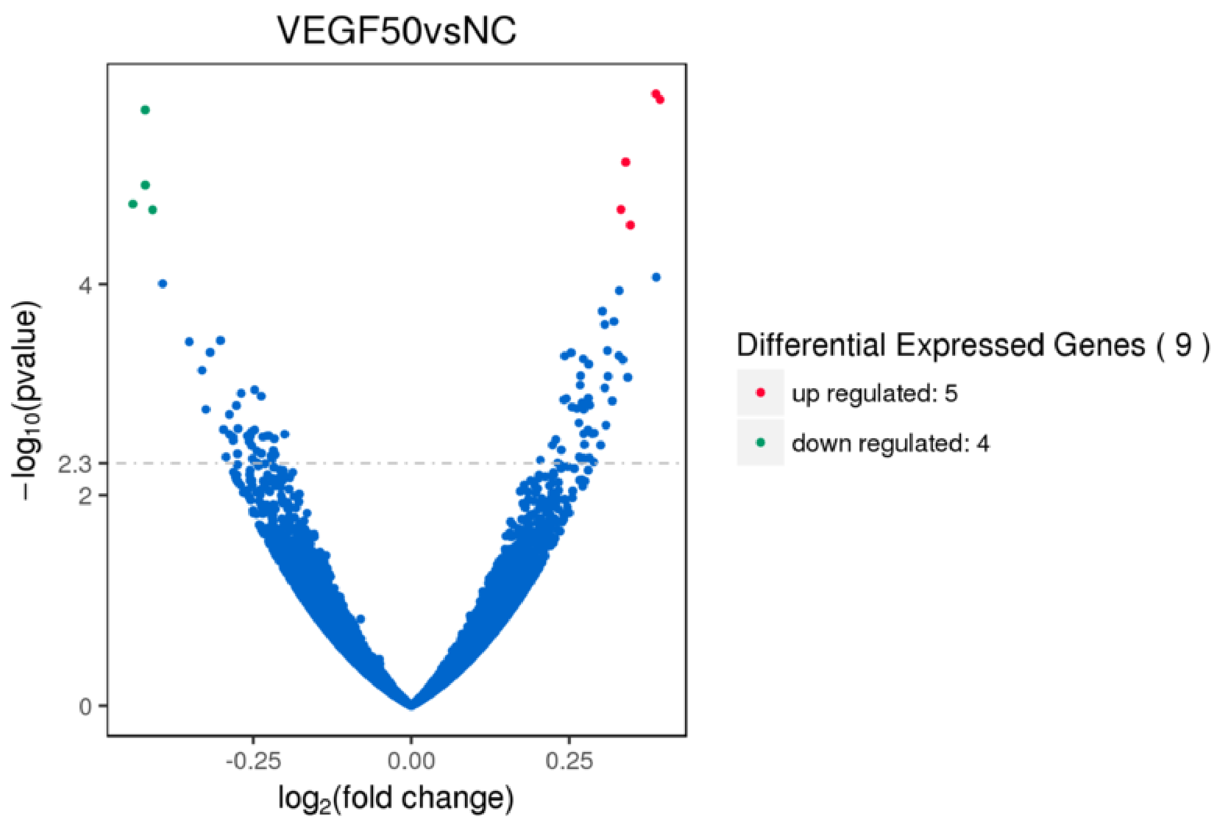

Figure 6. Volcano plot shows overall profile of VEGF-induced gene expression in human cervical epithelial cells. Of the total genes examined, nine were significantly expressed, of which five were up-regulated (red dot) and four were down-regulated (green dot) by VEGF (P-adjusted value <0.05; n=3).

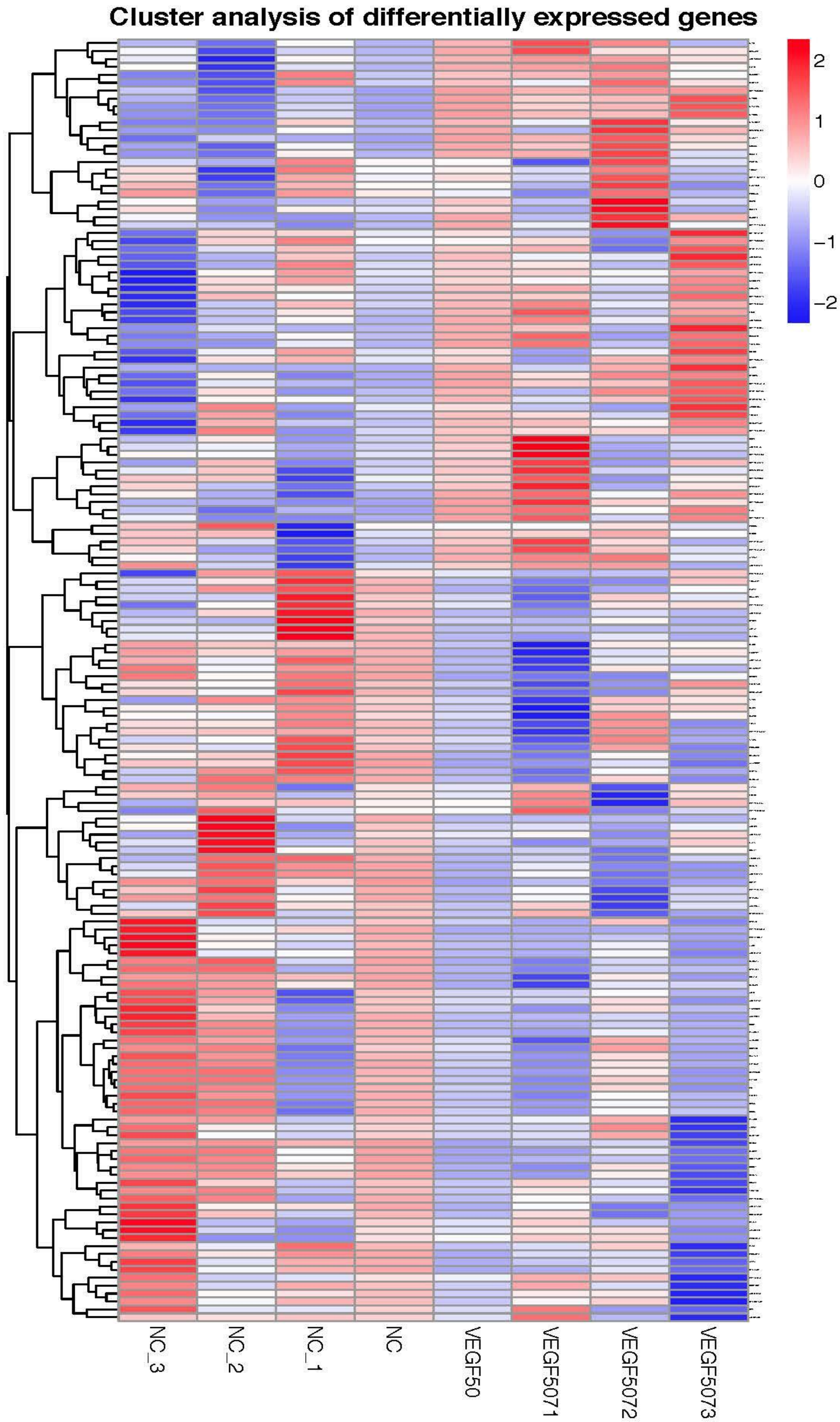

Figure 7. Heat map illustrating the effect of vascular endothelial growth factor (VEGF) on the gene expression profile of Human cervical epithelial cells versus vehicle (Negative control group) in culture.

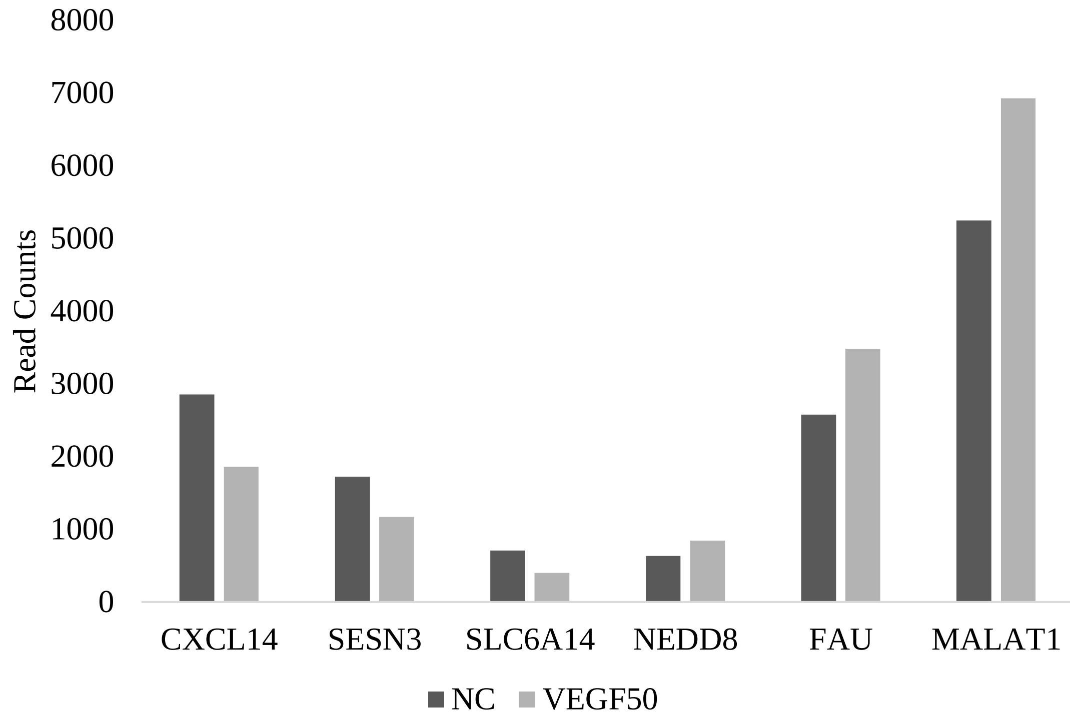

Figure 8. Vascular endothelial growth factor (VEGF) differentially alters expression of genes associated with proliferation in Human cervical epithelial cells. While VEGF significantly upregulates expression of three out of six genes (50%) associated with proliferation (*NEDD8, FAU, MALAT1*), it significantly downregulates the other half (C*XCL14*, *SESN3, SLC6A14*). P-adjusted value < 0.1, n=3.

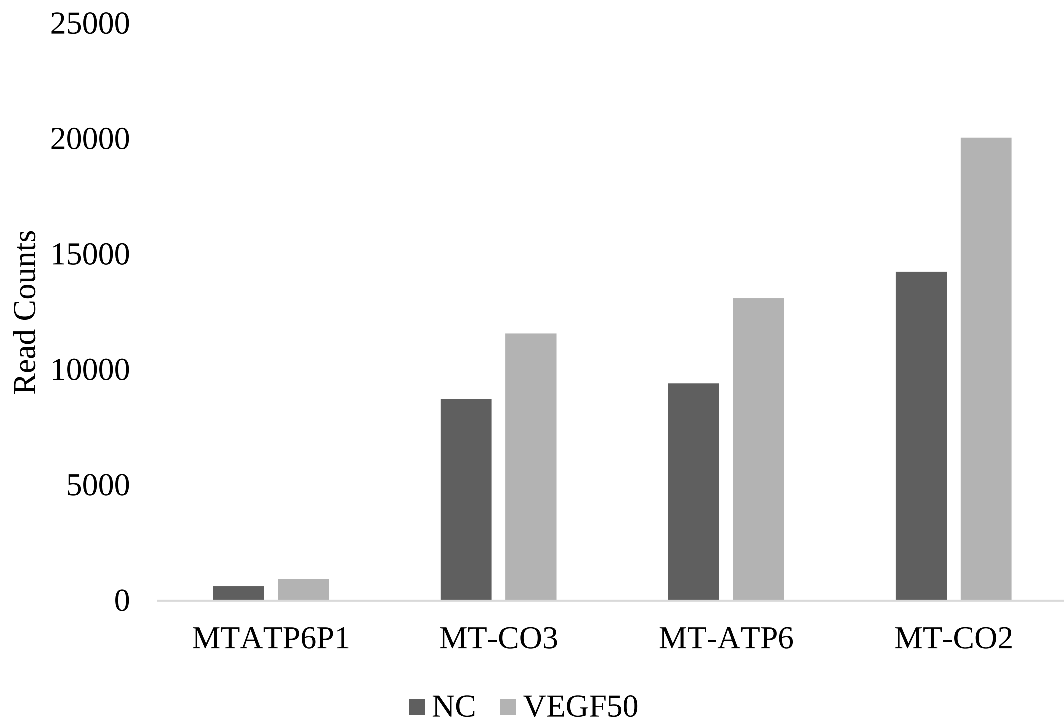

Figure 9. Vascular endothelial growth factor (VEGF) up regulates expression of genes associated with mitochondrial function in Human cervical epithelial cells. VEGF significantly upregulates expression of all the four genes associated with energy metabolism in human cervical epithelial cells (*MTATP6P1, MT-CO3, MT-ATP6, MT-C02*). P-adjusted value < 0.1, n=3.

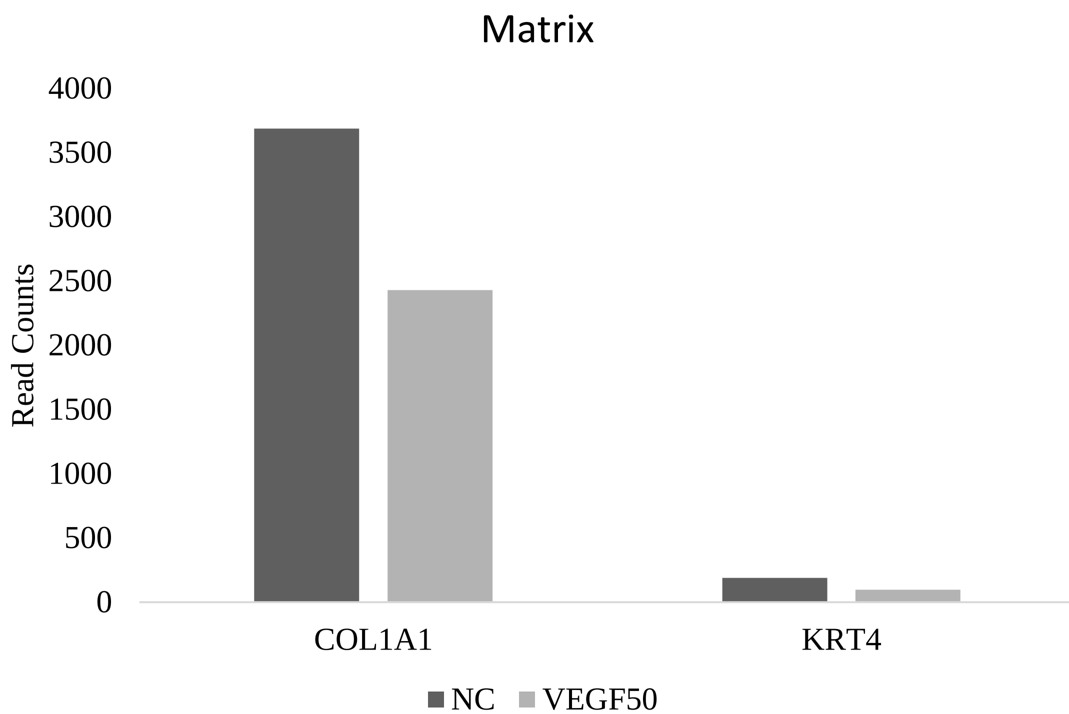

Figure 10. Vascular endothelial growth factor (VEGF) down regulates expression of extracellular matrix genes in Human cervical epithelial cells. VEGF significantly down regulates expression of genes associated with extracellular matrix, namely *COL1A1* and *KRT4*. P-adjusted value < 0.1, n=3.

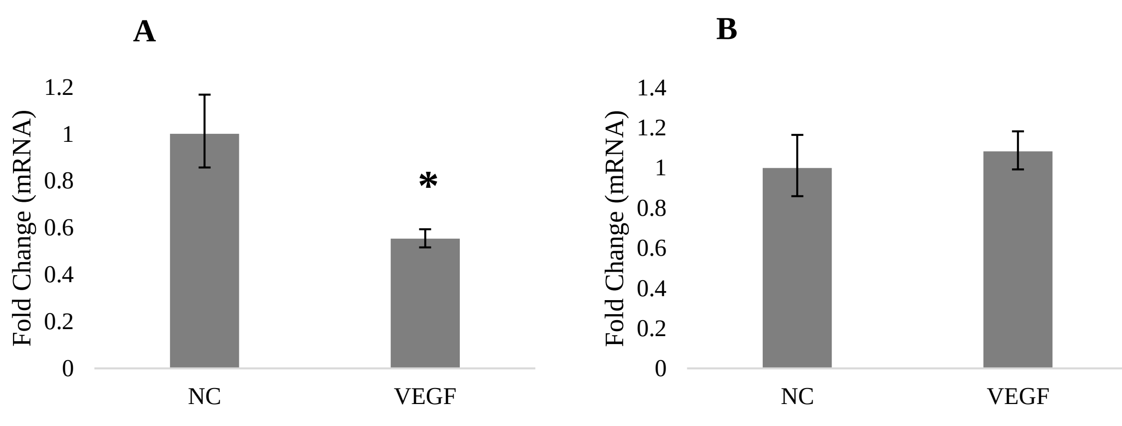

Figure 11. Verification of RNA-seq data by real-time PCR analysis. Treatment of Human cervical epithelial cells with VEGF leads to down regulation of SESN3 expression (A) and tends to up regulates MT-ATP6 (B), as revealed by RT-qPCR (n=4), *p<0.05. MT-ATP6 not statistically significant.

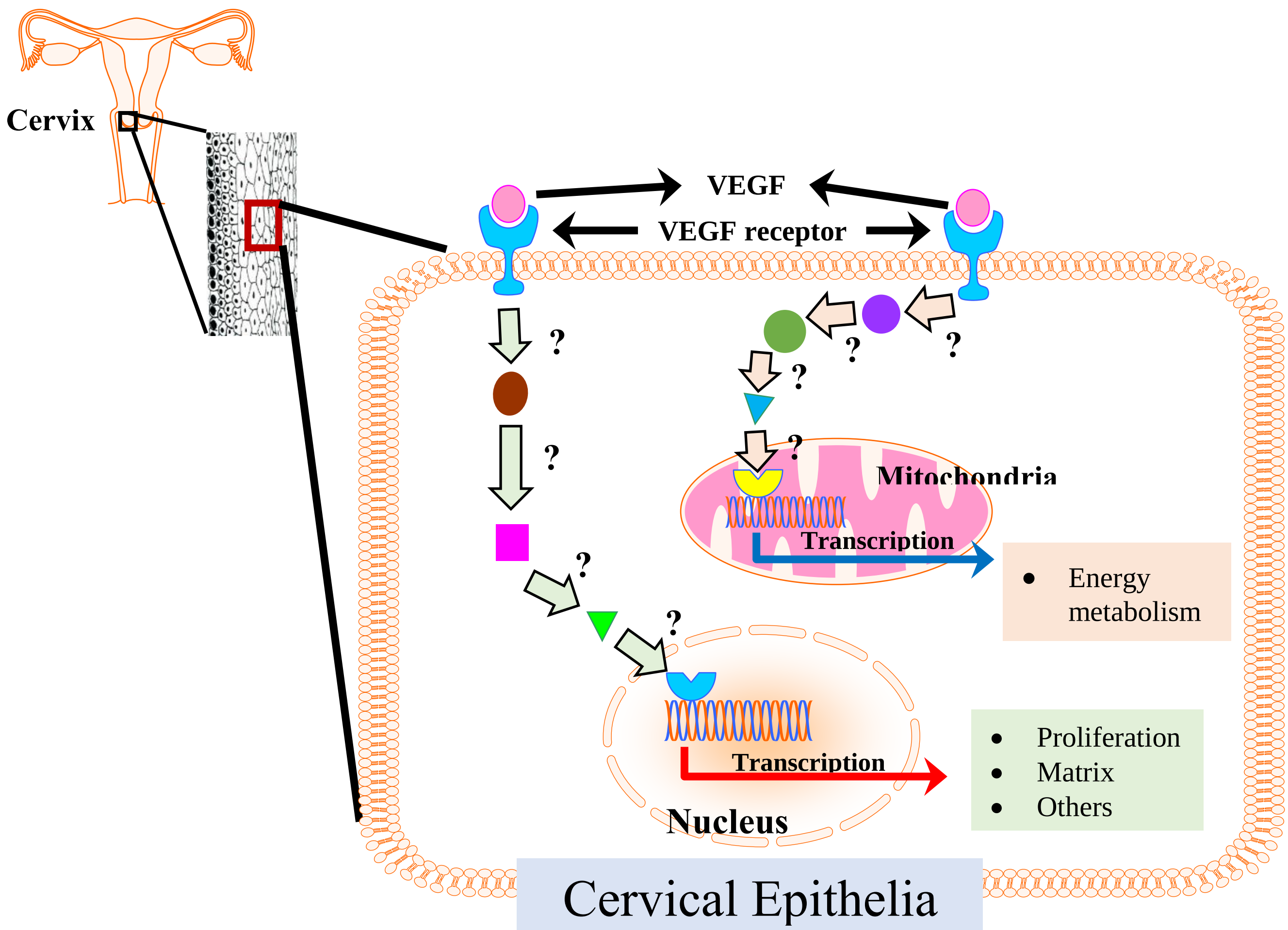

Figure 12. Proposed working model showing VEGF-regulated biological processes in Human cervical epithelial cells.

Table 1. Functional properties of the 162 VEGF-induced signature genes in Human cervical epithelial cells.

BIOLOGICAL GROUP	GENE NAME	LOG2 FOLD CHANGE
PROLIFERATION	V-MYB Myeloblastosis Viral Oncogene Homolog (*MYB*)	3.27
	Farnesyltransferase CAAX box Beta (*FNTB*)	6.43
	Metastasis Associated Lung Adenocarcinoma Transcript 1 (*MALAT1*)	0.33
	R-spondin 4 (*RSPO4*)	6.57
	Hect Domain and RLD 2 Pseudogene 10 (*HERC2P10*)	3.14
	Casein Kinase 2 Beta Polypeptide (*CSNK2B*)	1.36
	Apolipoprotein B mRNA Editing Enzyme Catalytic Polypeptide-like 3A (*APOBEC3A*)	6.25
	Apolipoprotein B mRNA Editing Enzyme Catalytic Polypeptide-like 3A (*FAU*)	0.35
	C-type Lectin Domain Family 3 Member A (*CLEC3A*)	-6.86
	STE20-related Kinase Adaptor Alpha (*STRADA*)	-2.12
	Ankyrin Repeat Domain 44 (*ANKRD44*)	-3.58
	Solute Carrier Family 6 (amino acid transporter) Member 14 (*SLC6A14*)	-0.44
	Transmembrane Protease Serine 13 (*TMPRSS13*)	-1.67
	Short Chain Dehydrogenase/Reductase Fmaily 9C Member 7 (*SDR9C7*)	-2.12
	Short Chain Dehydrogenase/Reductase Fmaily 9C Member 7 (*SESN3*)	-0.42
	TP3 Target 5 (*TP53TG5*)	-2.79
	Sclerostin Domain Containing 1 (*SOSTDC1*)	-3.10
	Chemokine (CXC Motif) Ligand 14	-0.42

	(*CXCL14*)	
IMMUNE RESPONSE	Caspase Recruitment Domain Family Member 17 (*CARD17*)	-6.25
	RP11-1112J20.2	2.71
	Dedicator of Cytokinesis 2 (*DOCK2*)	7.17
	Triggering Receptor Expressed on Myeloid Cells-like 2 (*TREML2*)	6.73
	CAP-GLY Domain Containing Linker Protein 3 (*CLIP3*)	-1.96
	Major Histocompatibility Complex Class II DQ Beta 1 (*HLA-DQB1*)	-1.37
	Chemokine (CXC Motif) Ligand 17 (*CXCL17*)	-1.84
	Lymphocyte-activation Gene 3 (*LAG3*)	-6.57
	Serpin Peptidase Inhibitor Clade B (ovalbumin) Member 3 (*SERPINB3*)	-1.28
	Peptidase Inhibitor 3 Skin-derived (*PI3*)	-1.50
	Cornulin (*CRNN*)	-2.29
	RP4-794H19.1	6.83
ENERGY METABOLISM	Mitochondrially Encoded Cytochrome C Oxidase II (*MT-C02*)	0.39
	Mitochondrially Encoded ATP Synthase 6 (*MT-ATP6*)	0.39
	Mitochondrially Encoded Cytochrome C Oxidase III (*MT-C03*)	0.34
	Succinate Dehydrogenase Complex Subunit A Flavoprotein Pseudogene 3 (*SDHAP3*)	2.92
	Proline Dehydrogenase (oxidase) 1 (*PRODH*)	6.66
	Translin-associated Factor X Interacting Protein 1 (*TSNAXIP1*)	-2.37
	Butyrobetaine (gamma) 2-Oxoglutarate (gamma-buyrobetaine hydroxylase) 1 (*BBOX1*)	-1.60
STRUCTURE/ MATRIX	Glycerophosphodiester Phospodiesterase Domain Containing 3 (*GDPD3*)	1.80
	Prostate Androgen-Regulated Mucin-like Protein 1 (*PARM1*)	-3.73
	Synaptonemal Complex Protein 2	-3.53

	(*SYCP2*)	
	Actin Alpha 1 Skeletal Muscle (*ACTA1*)	-6.28
	Actin Alpha 1 Skeletal Muscle (*TGM5*)	-3.81
	Matrix Metallopeptidase 24 (*MMP24*)	-1.67
	Matrix Metallopeptidase 24 (*LAMA4*)	-2.77
	Heparan Sulfate (Glucosamine) 3-O-Sulfotransferase 6 (*HS3ST6*)	-2.58
	Doublecortin-like Kinase 1 (*DCLK1*)	-6.83
	Collagen Type 1 Alpha 1 (*COL1A1*)	-0.41
	Keratinocyte Differentiation-associated Protein (*KRTDAP*)	-2.80
	Sp8 Transcription Factor (*SP8*)	-3.10
CELL ADHESION/ CELL-CELL COMMUNICATION	Protocadherin Gamma Subfamily B 2 (*PCDHGB2*)	2.93
	Carcinoembryonic Antigen-related Cell Adhesion Molecule 1 (biliary glycoprotein) (*CEACAM5*)	-1.74
	G Protein-coupled Receptor 124 (*GPR124*)	-2.47
	Desmoglein 1 (*DSG1*)	-2.09
	Suprabasin (*SBSN*)	-1.65
	Leucine Rich Repeat Neuronal 1 (*LRRN1*)	-2.71
	Cadherin 26 (*CDH26*)	-3.44
	Protocadherin Beta 13 (*PCDHB13*)	-4.10
NON-CODING RNA	*DLGAP1-AS1*	1.92
	RP11-617F23.1	6.40
	RP11-319G6.1	2.29
	RP13-188A5.1	3.04
	RP5-1057J7.6	6.39
	ZNF32-AS2	-6.28
	SRD5A3-AS1	-6.42
	LINC00452	-3.84
	CTC-523E23.1	-3.67

	RP11-373N22.3	-6.81
	DLG1-AS1	-6.57
	SOX21-AS1	-3.06
	STK4-AS1	-6.57
	RP1-46F2.2	-6.57
	STXBP5-AS1	-2.35
	HOXB-AS3	-6.69
PSEUDOGENE	*RP3-342P20.2*	2.73
	Cytochrome P450 Family 2 Subfamily B Polypeptide 7 Pseudogene 1 (*CYP2B7P1*)	6.54
	AC018755.16	2.21
	Formin Binding Protein 1 Pseudogene 1 (*FNBP1P1*)	-2.57
	SMAD Specific E3 Ubiquitin Protein Ligase 2 Pseudogene 1 (*SMURF2P1*)	-2.74
	RP11-764K9.4	-6.51
	Ribosomal Protein S26 Pseudogene 47 (*RPS26P47*)	-6.57
UNCHARACTERIZED	Ribosomal Protein SA Pseudogene 9 (*RPSAP9*)	6.43
	AC018738.2	6.69
	3 Oxoacid CoA Transferase 2 Pseudogene 1 (*OXCT2P1*)	2.79
	RP11-536C5.7	6.43
	Myotubularin Related Protein 9-like Pseudogene (*MTMR9LP*)	-6.51
	RP3-395M20.2	2.48
	Chromosome 8 open reading frame 56 (*C8orf47*)	3.72
	Polycystic Kidney Disease 1 (autosomal dominant) Pseudogene 5 (*PKD1P5*)	-1.43
	Tripartite Motif Containing 61 (*TRIM61*)	2.71
	RP11-46H11.12	3.82
	Family with Sequence Similarity 186 Member B (*FAM186B*)	3.82
	RP11-305N23.1	6.51
	CTC-471J1.10	6.25
	AC006273.5	6.39
	AC132872.2	3.76
	RP11-247L20.4	2.43
	RP11-127120.4	3.58

	Transmembrane Protein 256 (*TMEM256*)	1.71
	RP11-252A24.2	1.84
	CTC-436P18.3	3.58
	CTD-2521M24.6	6.51
	RP11-264B17.2	2.37
	AC012314.8	6.66
	RP1-266L20.9	3.96
	RP11-666A8.7	6.54
	RP11-320L11.2	6.66
	RP11-540A21.2	3.77
	RP11-505K9.1	-3.53
	AC016700.5	-6.54
	RP11-1212A22.1	-1.68
	FOXD2 Antisense RNA 1 (*FOXD2*)	-6.92
	AC018642.1	-1.84
	AC010761.13	-6.25
	RP11-17M16.2	-2.89
	AC087491.2	-6.57
	AC012313.1	-2.35
	Ankyrin Repeat Domain 22 (*ANKRD22*)	-1.54
	Ankyrin Repeat Domain 22 (*GABRP*)	-3.18
	RP11-265B8.4	-4.53
	AC046143.3	-2.26
	AC025627.9	-1.82
OTHERS/ MISCELLANEOUS	Ras Protein-specific Guanine Nucleotide-releasing Factor 1 (*RASGRF1*)	6.57
	Potassium Voltage-gated Channel Subfamily H (eag-related) Member 3 (*KCNH3*)	3.35
	Protocadherin Alpha 3 (*PCDHA3*)	2.93
	Ring Finger Protein 212 (*RNF212*)	3.19
	Retinol Binding Protein 7 Cellular (*RBP7*)	6.40
	RP1-90J20.12	6.54
	ATPase Ca++ Transporting Cardiac Muscle Fast Twitch 1 (*ATP2A1*)	3.77
	DNAJ (Hsp40) Homolog Subfamily C Member 12 (*DNAJC12*)	-3.81

	GLIS Family Zinc Finger 2 (*GLIS2*)	-1.45
	GLIS Family Zinc Finger 2 (*HIST1H2AI*)	-3.63
	Cysteine-rich Secretory Protein 3 (*CRISP3*)	-3.05
	Myelin Regulatory Factor (*MYRF*)	-6.54
	Calcyphosine 2 (*CAPS2*)	-2.97
	Calcyphosine 2 (*SLC34A2*)	-1.79
	Microseminoprotein Beta (*MSMB*)	-2.92
	Kallikrein-related Peptidase 14 (*KLK14*)	-3.76
	Cytochrome P450 Family 4 Subfamily F Polypeptide 3 (*CYP4F3*)	-3.90
	P3-410C9.1	-3.82
	Cytochrome P450 Family 4 Subfamily F Polypeptide 22 (*CYP4F22*)	-1.71
	Discs Large (Drosophila) Homolog-associated Protein 3 (*DLGAP3*)	-6.42
	HOP Homeobox (*HOPX*)	-1.32
	Phospholipase A2 Gropu IVE (*PLA2G4E*)	-2.51
	Aldehyde Dehydrogenase 3 Fmaily Member B2 (*ALDH3B2*)	-1.44
	Calmodulin-like 5 (*CALML5*)	-1.31
	Fetuin B (*FETUB*)	-2.53
	Zinc Finger Protein 750 (*ZNF750*)	-1.26
	Phospholipase A2 Group VII (platelet-activating factor acetylhydrolase plasma) (*PLA2G7*)	-2.09
	CDKN2B-Antisense RNA 1 (*CDKN2B-AS1*)	-6.25
	Small Integral Membrane Protein 6 (*SMIM6*)	-3.71
	Rho Guanine Nucleotide Exchange Factor (GEF) 33 (*ARHGEF33*)	-3.71

	Relaxin 2 (*RLN2*)	-6.57
	Hephaestin (*HEPH*)	-3.71
	AC012314.8	0.03
	AC018766.5	0.08
	BTB_(POZ) (BTBD16	0.09
	Lin-37 homolog (LIN37)	0.08
	Mucin 12 Cell Surface Associated (MUC12)	0.06
	RP11-265B8.4	-0.09
	RP11-336A10.4	0.02
	RP11-536C5.7	0.08
	RP11-552M11.4	0.03
	RP11-767N6.7	0.14
	RP11-867G23.3	-0.01

Table 2. Transcriptomic profile of vascular endothelial growth factor (VEGF)-induced proliferative signature genes in Human cervical epithelial cells. Only the gene expression of the first six genes in the Table were significantly altered.

GENE	NC READ COUNT	VEGF50 READ COUNT	FOLD CHANGE	P-ADJ VALUE	UP/DOWN
1. SESN3	1725.83	1171.06	-0.42	**0.008**	Down
2. CXCL14	2852.78	1858.32	-0.42	**0.024**	Down
3. SLC6A	708.78	401.75	-0.44	**0.025**	Down
4. MALAT1	5246.02	6927.50	0.33	**0.025**	Up
5. FAU	2578.77	3485.36	0.35	**0.031**	Up
6. NEDD8	631.45	846.48	0.33	**0.099**	Up
7. MYB	6.07	15.62	0.09	NA	Up
8. FNTB	3.77	8.48	0.05	NA	Up
9. RSPO4	1.38	4.74	0.04	NA	Up
10. HERC2P10	11.27	14.86	0.03	NA	Up
11. CSNK2B	63.29	105.16	0.22	NA	Up
12. APOBEC3A	2.11	3.39	0.01	NA	Up
13. CLEC3A	7.32	1.33	0.06	NA	Up
14. STRADA	31.25	17.40	-0.13	NA	Down
15. ANKRD44	9.30	5.39	-0.04	NA	Down
16. TMPRSS13	62.74	37.78	-0.12	NA	Down
17. SDR9C7	25.78	15.16	-0.06	NA	Down
18. TP53TG5	16.25	9.42	-0.07	NA	Down
19. SOSTDC1	13.29	8.45	-0.05	NA	Down

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Vita

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MacKinsey was Co-President of the Biology Graduate Student Association as well as an Introductory Biology Lab instructor during her time as a graduate student. She plans to pursue a career as a Physician's Assistant with a focus in women's reproductive health.